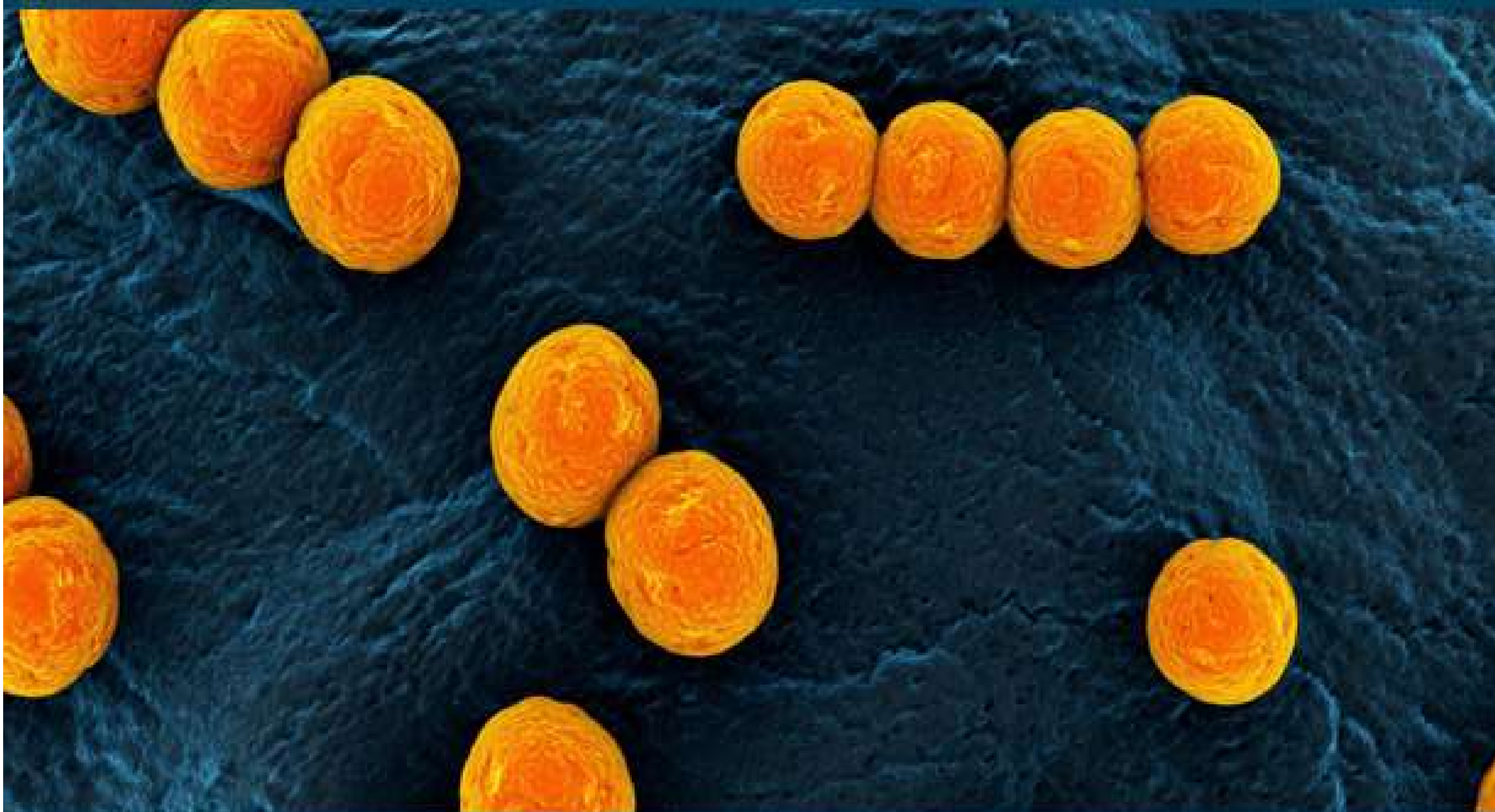
Elements, Essence, Techniques and Applications of Microbiology



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Chapter 1

Food Microbiology

Food microbiology is the study of the microorganisms that inhabit, create, or contaminate food. Of major importance is the study of microorganisms causing food spoilage. "Good" bacteria, however, such as probiotics, are becoming increasingly important in food science. In addition, microorganisms are essential for the production of foods such as cheese, yogurt, other fermented foods, bread, beer and wine.

Food safety

Food safety is a major focus of food microbiology. Pathogenic bacteria, viruses and toxins produced by microorganisms are all possible contaminants of food. However, microorganisms and their products can also be used to combat these pathogenic microbes. Probiotic bacteria, including those that produce bacteriocins, can kill and inhibit pathogens. Alternatively, purified bacteriocins such as nisin can be added directly to food products. Finally, bacteriophages, viruses that only infect bacteria, can be used to kill bacterial pathogens. Thorough preparation of food, including proper cooking, eliminates most bacteria and viruses. However, toxins produced by contaminants may not be heat-labile, and some are not eliminated by cooking.

Fermentation

Fermentation is one way microorganisms can change a food. Yeast, especially *Saccharomyces cerevisiae*, is used to leaven bread, brew beer and make wine. Certain bacteria, including lactic acid bacteria, are used to make yogurt, cheese, hot sauce, pickles, fermented sausages and dishes such as kimchi. A common effect of these fermentations is that the food product is less hospitable to other microorganisms, including pathogens and spoilage-causing microorganisms, thus extending the food's shelf-life.

Food fermentations are ancient technologies that harness microorganisms and their enzymes to improve the human diet. Fermented foods keep better, have enhanced flavours, textures and aromas, and may also possess certain health benefits, including

superior digestibility. For vegetarians, fermented foods serve as palatable, protein-rich meat substitutes.

Some cheese varieties also require molds to ripen and develop their characteristic flavors.

Asian cuisines rely on a large repertoire of fermented foods. In particular, *Aspergillus oryzae* and *A. sojae*, sometimes called koji molds, are employed in many ways. Their hydrolytic enzymes suit them for growth on starch and other carbohydrate-rich substrates. In the koji process, fungal enzymes perform the same function as the malting enzymes used in the beer fermentations of western cultures. The koji molds release amylases that break down rice starch, which in turn can be fermented to make rice wine. Fermented rice beverages have numerous local variations and names, depending on country and region. Rice wine is called *shaoshing* in parts of China, *sake* in Japan, *takj* or *yakju* in Korea, as well as by many other names across Asia. The koji molds are also effective in a variety of legume fermentations, of which miso and soy sauce are best known. Miso is a mixture of soybeans and cereals usually used to flavour soups. Soy sauce is a flavourful, salty liquid sauce made from soybeans that have been fermented by koji molds, yeasts, as well as several halophilic bacteria. Other names for soy sauce include *jiangyou* (China), *makjang* and *kanjang* (Korea), *toyo* (Philippines) and *siiu* (Thailand).

Probiotics

Probiotics are living organisms that, when consumed, have beneficial health benefits outside their inherent nutritional effects. There is a growing body of evidence for the role of probiotics in gastrointestinal infections, irritable bowel syndrome and inflammatory bowel disease.

Lactobacillus species are used for the production of yogurt, cheese, sauerkraut, pickles, beer, wine, cider, kimchi, chocolate and other fermented foods, as well as animal feeds such as silage. In recent years, much interest has been shown in the use of lactobacilli as probiotic organisms and their potential for disease prevention in humans and animals.

Bifidobacteria are considered as important probiotics, and are used in the food industry to relieve and treat many intestinal disorders. Bifidobacteria exert a range of beneficial health effects, including the regulation of intestinal microbial homeostasis, the inhibition of pathogens and harmful bacteria that colonize and/or infect the gut mucosa, the modulation of local and systemic immune responses, the repression of procarcinogenic enzymatic activities within the microbiota, the production of vitamins, and the bioconversion of a number of dietary compounds into bioactive molecules.

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Microbial biopolymers

A variety of biopolymers, such as polysaccharides, polyesters and polyamides, are naturally produced by microorganisms. Several microbially-produced polymers are used in the food industry.

Xanthan

Plant-pathogenic bacteria of the genus *Xanthomonas* are able to produce the acidic exopolysaccharide xanthan gum. Because of its physical properties, it is widely used as a viscosifer, thickener, emulsifier or stabilizer in the food industry. Xanthan consists of pentasaccharide repeat units composed of D-glucosyl, D-mannosyl, and D-glucuronyl acid residues in a molar ratio of 2:2:1 and variable proportions of O-acetyl and pyruvyl residues.

Alginate

Alginate is the main representative of a family of polysaccharides that neither show branching nor repeating blocks or unit patterns and this property distinguishes it from to other polymers like xanthan or dextran. Alginates can be used as thickening agents. Although listed here under the category 'Microbial polysaccharides', commercial alginates are currently only produced by extraction from brown seaweeds such as *Laminaria hyperborea* or *L. japonica*.

Cellulose

Cellulose is a simple polysaccharide, in that it consists only of one type of sugar (glucose), and the units are linearly arranged and linked together by β-1,4 linkages only. The mechanism of biosynthesis is, however, rather complex, partly because in native celluloses, the chains are organized as highly ordered water-insoluble fibers. Currently, the key genes involved in cellulose biosynthesis and regulation are known in a number of bacteria, but many details of the biochemistry of its biosynthesis are still not clear. In spite of the enormous abundance of cellulose in plants, bacterial celluloses are being investigated for industrial exploitations.

Poly-γ-glutamic acid

Poly-γ-glutamic acid (γ-PGA) produced by various strains of *Bacillus* has potential applications as a thickener in the food industry.

Levan

Levan, a homopolysaccharide composed of D-fructofuranosyl residues joined by 2,6 with multiple branches by 2,1 linkages, has great potential as a functional biopolymer in foods,

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feeds, cosmetics, and the pharmaceutical and chemical industries. Levan can be used as food or a feed additive with prebiotic and hypocholesterolemic effects.

Exopolysaccharides

Microorganisms synthesize a wide spectrum of multifunctional polysaccharides, including intracellular polysaccharides, structural polysaccharides and extracellular polysaccharides or exopolysaccharides (EPSs). EPSs generally consist of monosaccharides and some noncarbohydrate substituents (such as acetate, pyruvate, succinate, and phosphate). Owing to the wide diversity in composition, they have found multifarious applications in various food and pharmaceutical industries.

Foodborne pathogens

Foodborne pathogens are the leading causes of illness and death in less developed countries, killing approximately 1.8 million people annually. In developed countries, foodborne pathogens are responsible for millions of cases of infectious gastrointestinal diseases each year, costing billions of dollars in medical care and lost productivity. New foodborne pathogens and foodborne diseases are likely to emerge, driven by factors such as pathogen evolution, changes in agricultural and food manufacturing practices, and changes to the human host status. There are growing concerns that terrorists could use pathogens to contaminate food and water supplies in attempts to incapacitate thousands of people and disrupt economic growth.

Enteric viruses

Food and waterborne viruses contribute to a substantial number of illnesses throughout the world. Among those most commonly known are hepatitis A virus, rotavirus, astrovirus, enteric adenovirus, hepatitis E virus, and the human caliciviruses consisting of the noroviruses and the Sapporo viruses. This diverse group is transmitted by the fecal-oral route, often by ingestion of contaminated food and water.

Protozoan parasites

Protozoan parasites associated with food and water can cause illness in humans. Although parasites are more commonly found in developing countries, developed countries have also experienced several foodborne outbreaks. Contaminants may be inadvertently introduced to the foods by inadequate handling practices, either on the farm or during processing of foods. Protozoan parasites can be found worldwide, either infecting wild animals or in water and contaminating crops grown for human consumption. The disease can be much more severe and prolonged in immunocompromissed individuals.

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Mycotoxins

Molds produce mycotoxins, which are secondary metabolites that can cause acute or chronic diseases in humans when ingested from contaminated foods. Potential diseases include cancers and tumors in different organs (heart, liver, kidney, nerves), gastrointestinal disturbances, alteration of the immune system, and reproductive problems. Species of *Aspergillus*, *Fusarium*, *Penicillium*, and *Claviceps* grow in agricultural commodities or foods and produce the mycotoxins such as aflatoxins, deoxynivalenol, ochratoxin A, fumonisins, ergot alkaloids, T-2 toxin, and zearalenone and other minor mycotoxins such as cyclopiazonic acid and patulin. Mycotoxins occur mainly in cereal grains (barley, maize, rye, wheat), coffee, dairy products, fruits, nuts and spices. Control of mycotoxins in foods has focused on minimizing mycotoxin production in the field, during storage or destruction once produced. Monitoring foods for mycotoxins is important to manage strategies such as regulations and guidelines, which are used by 77 countries, and for developing exposure assessments essential for accurate risk characterization.

Aflatoxins are still recognized as the most important mycotoxins. They are synthesized by only a few *Aspergillus* species, of which *A. flavus* and *A. parasiticus* are the most problematic. The expression of aflatoxin-related diseases is influenced by factors such as age, nutrition, sex, species and the possibility of concurrent exposure to other toxins. The main target organ in mammals is the liver, so aflatoxicosis is primarily a hepatic disease. Conditions increasing the likelihood of aflatoxicosis in humans include limited availability of food, environmental conditions that favor mold growth on foodstuffs, and lack of regulatory systems for aflatoxin monitoring and control.

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Yersinia enterocolitica

Yersinia enterocolitica includes pathogens and environmental strains that are ubiquitous in terrestrial and fresh water ecosystems. Evidence from large outbreaks of yersiniosis and from epidemiological studies of sporadic cases has shown that *Y. enterocolitica* is a foodborne pathogen. Pork is often implicated as the source of infection. The pig is the only animal consumed by man that regularly harbors pathogenic *Y. enterocolitica*. An important property of the bacterium is its ability to multiply at temperatures near 0°C, and therefore in many chilled foods. The pathogenic serovars (mainly O:3, O:5,27, O:8 and O:9) show different geographical distribution. However, the appearance of strains of serovars O:3 and O:9 in Europe, Japan in the 1970s, and in North America by the end of the 1980s, is an example of a global pandemic. There is a possible risk of reactive arthritis following infection with *Y. enterocolitica*.

Vibrio

Vibrio species are prevalent in estuarine and marine environments, and seven species can cause foodborne infections associated with seafood. *Vibrio cholerae* O1 and O139 serovtypes produce cholera toxin and are agents of cholera. However, fecal-oral route infections in the terrestrial environment are responsible for epidemic cholera. *V. cholerae*

non-O1/O139 strains may cause gastroenteritis through production of known toxins or unknown mechanism. *Vibrio parahaemolytitcus* strains capable of producing thermostable direct hemolysin (TDH) and/or TDH-related hemolysin are most important causes of gastroenteritis associated with seafood consumption. *Vibrio vulnificus* is responsible for seafoodborne primary septicemia, and its infectivity depends primarily on the risk factors of the host. *V. vulnificus* infection has the highest case fatality rate (50%) of any foodborne pathogen. Four other species (*V. mimicus*, *V. hollisae*, *V. fluvialis*, and *V. furnissii*) can cause gastroenteritis. Some strains of these species produce known toxins, but the pathogenic mechanism is largely not understood. The ecology of and detection and control methods for all seafoodborne *Vibrio* pathogens are essentially similar.

Staphylococcus aureus

Staphylococcus aureus is a common cause of bacterial foodborne disease worldwide. Symptoms include vomiting and diarrhea that occur shortly after ingestion of *S. aureus* toxin-contaminated food. The symptoms arise from ingestion of preformed enterotoxin, which accounts for the short incubation time. Staphylococcal enterotoxins are superantigens and, as such, have adverse effects on the immune system. The enterotoxin genes are accessory genetic elements in *S. aureus*, meaning not all strains of this organism are enterotoxin-producing. The enterotoxin genes are found on prophages, plasmids, and pathogenicity islands in different strains of *S. aureus*. Expression of the enterotoxin genes is often under the control of global virulence gene regulatory systems.

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Campylobacter

Campylobacter spp., primarily *C. jejuni* subsp. *jejuni* is one of the major causes of bacterial gastroenteritis in the U.S. and worldwide. *Campylobacter* infection is primarily a foodborne illness, usually without complications; however, serious sequelae, such as Guillain-Barre Syndrome, occur in a small subset of infected patients. Detection of *C. jejuni* in clinical samples is readily accomplished by culture and nonculture methods.

Listeria monocytogenes

Listeria monocytogenes is Gram-positive foodborne bacterial pathogen and the causative agent of human listeriosis. *Listeria* infections are acquired primarily through the consumption of contaminated foods, including soft cheese, raw milk, deli salads, and ready-to-eat foods such as luncheon meats and frankfurters. Although *L. monocytogenes* infection is usually limited to individuals that are immunocompromised, the high mortality rate associated with human listeriosis makes it the leading cause of death among foodborne bacterial pathogens. As a result, tremendous effort has been made to develop methods for the isolation, detection and control of *L. monocytogenes* in foods.

Salmonella

Salmonella serotypes continue to be a prominent threat to food safety worldwide. Infections are commonly acquired by animal to human transmission though consumption of undercooked food products derived from livestock or domestic fowl. The second half of the 20th century saw the emergence of *Salmonella* serotypes that became associated with new food sources (i.e. chicken eggs) and the emergence of *Salmonella* serotypes with resistance against multiple antibiotics.

Shigella

Shigella species are members of the family Enterobacteriaceae and are Gram negative, nonmotile rods. Four subgroups exist based on O-antigen structure and biochemical properties: *S. dysenteriae* (subgroup A), *S. flexneri* (subgroup B), *S. boydii* (subgroup C) and *S. sonnei* (subgroup D). Symptoms include mild to severe diarrhea with or without blood, fever, tenesmus and abdominal pain. Further complications of the disease may be seizures, toxic megacolon, reactive arthritis and hemolytic uremic syndrome. Transmission of the pathogen is by the fecal-oral route, commonly through food and water. The infectious dose ranges from 10-100 organisms. *Shigella* spp. have a sophisticated pathogenic mechanism to invade colonic epithelial cells of the host, man and higher primates, and the ability to multiply intracellularly and spread from cell to adjacent cell via actin polymerization. *Shigella* spp. are one of the leading causes of bacterial foodborne illnesses and can spread quickly within a population.

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Escherichia coli

More information is available concerning *Escherichia coli* than any other organism, thus making *E. coli* the most thoroughly studied species in the microbial world. For many years, *E. coli* was considered a commensal of human and animal intestinal tracts with low virulence potential. It is now known that many strains of *E. coli* act as pathogens, inducing serious gastrointestinal diseases and even death in humans. There are six major categories of *E. coli* strains that cause enteric diseases in humans, including the (1) enterohemorrhagic *E. coli*, which cause hemorrhagic colitis and hemolytic uremic syndrome, (2) enterotoxigenic *E. coli*, which induce traveler's diarrhea, (3) enteropathogenic *E. coli*, which cause a persistent diarrhea in children living in developing countries, (4) enteroaggregative *E. coli*, which provokes diarrhea in children, (5) enteroinvasive *E. coli* that are biochemically and genetically related to *Shigella* species and can induce diarrhea, and (6) diffusely adherent *E. coli*, which cause diarrhea and are distinguished by a characteristic type of adherence to mammalian cells.

Clostridium botulinum and *Clostridium perfringens*

Clostridium botulinum produces extremely potent neurotoxins that result in the severe neuroparalytic disease, botulism. The enterotoxin produced by *C. perfringens* during sporulation of vegetative cells in the host intestine results in debilitating acute diarrhea and abdominal pain. Sales of refrigerated, processed foods of extended durability

including sous-vide foods, chilled ready-to-eat meals, and cook-chill foods have increased over recent years. Anaerobic spore-formers have been identified as the primary microbiological concerns in these foods. Heightened awareness over intentional food source tampering with botulinum neurotoxin has arisen with respect to genes encoding the toxins that are capable of transfer to nontoxigenic clostridia.

Bacillus cereus

The *Bacillus cereus* group comprises six members: *B. anthracis*, *B. cereus*, *B. mycoides*, *B. pseudomycoides*, *B. thuringiensis* and *B. weihenstephanensis*. These species are closely related and should be placed within one species, except for *B. anthracis* that possesses specific large virulence plasmids. *B. cereus* is a normal soil inhabitant, and is frequently isolated from a variety of foods, including vegetables, dairy products and meat. It causes a vomiting or diarrhea illness that is becoming increasingly important in the industrialized world. Some patients may experience both types of illness simultaneously. The diarrheal type of illness is most prevalent in the western hemisphere, whereas the emetic type is most prevalent in Japan. Desserts, meat dishes, and dairy products are the foods most frequently associated with diarrheal illness, whereas rice and pasta are the most common vehicles of emetic illness. The emetic toxin (cereulide) has been isolated and characterized; it is a small ring peptide synthesised nonribosomally by a peptide synthetase. Three types of *B. cereus* enterotoxins involved in foodborne outbreaks have been identified. Two of these enterotoxins are three-component proteins and are related, while the last is a one-component protein (CytK). Deaths have been recorded both by strains that produce the emetic toxin and by a strain producing only CytK. Some strains of the *B. cereus* group are able to grow at refrigeration temperatures. These variants raise concern about the safety of cooked, refrigerated foods with an extended shelf life. *B. cereus* spores adhere to many surfaces and survive normal washing and disinfection (except for hypochlorite and UVC) procedures. *B. cereus* food borne illness is likely under-reported because of its relatively mild symptoms, which are of short duration.

Food authenticity

It is important to be able to detect microorganisms in food, in particular pathogenic microorganisms or genetically modified microorganisms. Real-time PCR is an accepted analytical tool within the food industry. Its principal role has been one of assisting the legislative authorities, major manufacturers and retailers to confirm the authenticity of foods. The most obvious role is the detection of genetically modified organisms, but real-time PCR makes a significant contribution to other areas of the food industry, including food safety.

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Chapter 2

Algae

Algae

Laurencia, a marine genus of Red Algae from Hawaii.

Scientific classification

Domain: Eukaryota

Included groups

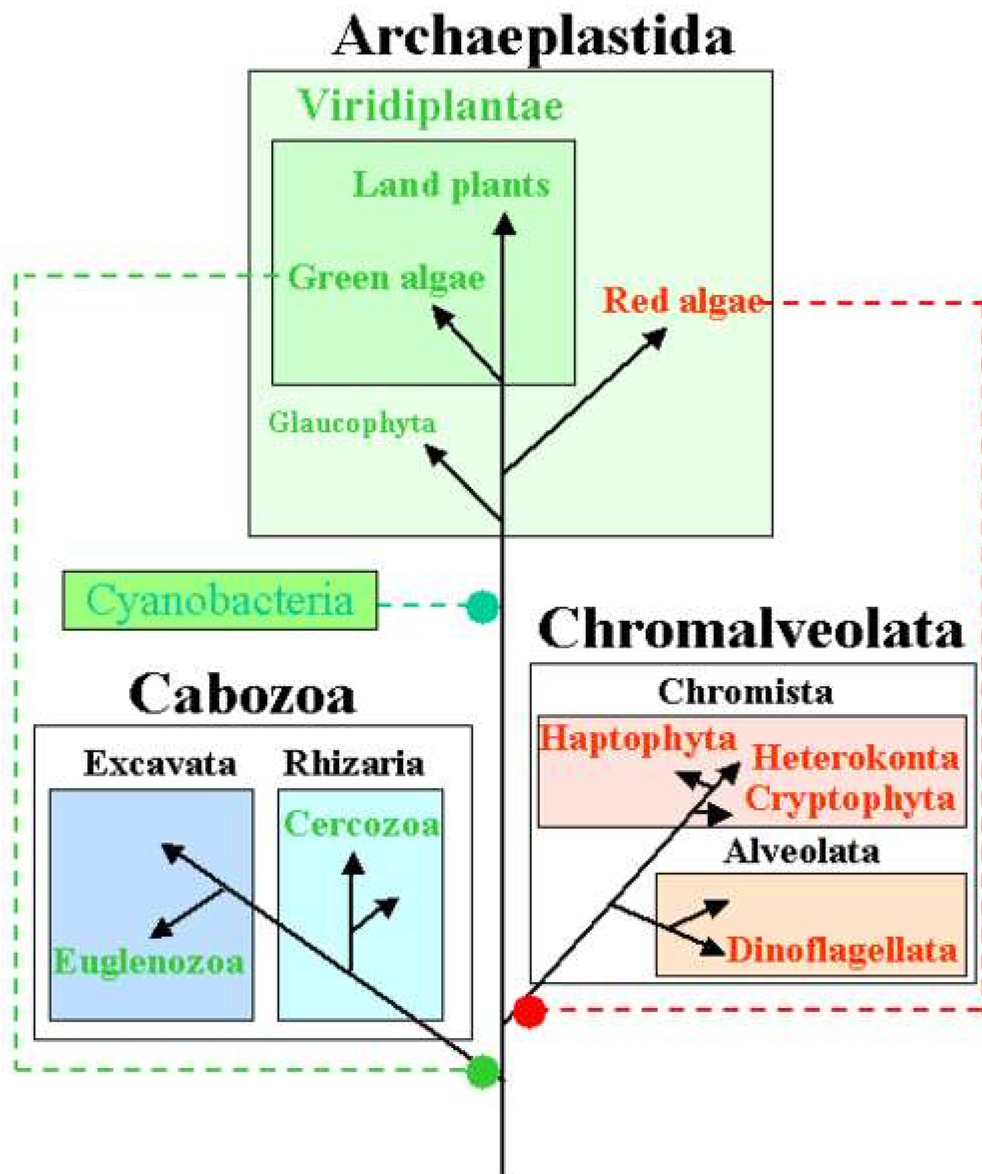
- Archaeplastida
  - Chlorophyta (Green algae)
  - Rhodophyta (Red algae)
  - Glaucophyta
- Rhizaria, Excavata
  - Chlorarachniophytes
  - Euglenids
- Chromista, Alveolata
  - Heterokonts
    - Bacillariophyceae (Diatoms)
    - Axodine
    - Bolidomonas
    - Eustigmatophyceae
    - Phaeophyceae (Brown algae)

- Chrysophyceae (Golden algae)
- Raphidophyceae
- Synurophyceae
- Xanthophyceae (Yellow-green algae)

- Cryptophyta
- Dinoflagellates
- Haptophyta

Excluded groups

- Cyanobacteria
- Plantae



The lineage of algae according to Thomas Cavalier-Smith. The exact number and placement of endosymbiotic events is not yet clear, so this diagram can be taken only as a general guide It represents the most parsimonious way of explaining the three types of endosymbiotic origins of plastids. These types include the endosymbiotic events of cyanobacteria, red algae and green algae, leading to the hypothesis of the supergroups Archaeplastida, Chromalveolata and Cabozoa respectively. However, the monophyly of Cabozoa has been refuted and the monophylies of Archaeplastida and Chromalveolata are currently strongly challenged. Endosymbiotic events are noted by dotted lines.

Algae are a large and diverse group of simple, typically autotrophic organisms, ranging from unicellular to multicellular forms, such as the giant kelps that grow to 65 meters in length. The US Algal Collection is represented by almost 300,000 accessioned and inventoried herbarium specimens. The largest and most complex marine forms are called seaweeds. They are photosynthetic like plants, and "simple" because their tissues are not organized into the many distinct organs found in land plants.

Though the prokaryotic cyanobacteria (commonly referred to as blue-green algae) were traditionally included as "algae" in older textbooks, many modern sources regard this as outdated as they are now considered to be bacteria. The term *algae* is now restricted to eukaryotic organisms. All true algae therefore have a nucleus enclosed within a membrane and plastids bound in one or more membranes. Algae constitute a paraphyletic and polyphyletic group, as they do not include all the descendants of the last universal ancestor nor do they all descend from a common algal ancestor, although their plastids seem to have a single origin. Diatoms are also examples of algae.

Algae are found in the fossil record dating back to approximately 3 billion years in the Precambrian. They exhibit a wide range of reproductive strategies, from simple, asexual cell division to complex forms of sexual reproduction.

Algae lack the various structures that characterize land plants, such as phyllids (leaves) and rhizoids in nonvascular plants, or leaves, roots, and other organs that are found in tracheophytes (vascular plants). Many are photoautotrophic, although some groups contain members that are mixotrophic, deriving energy both from photosynthesis and uptake of organic carbon either by osmotrophy, myzotrophy, or phagotrophy. Some unicellular species rely entirely on external energy sources and have limited or no photosynthetic apparatus.

Nearly all algae have photosynthetic machinery ultimately derived from the Cyanobacteria, and so produce oxygen as a by-product of photosynthesis, unlike other photosynthetic bacteria such as purple and green sulfur bacteria. Fossilized filamentous algae from the Vindhya basin have been dated back to 1.6 to 1.7 billion years ago.

Etymology and study

HISTORIA

FVCORVM

AVCTORE

SAMVEL GOTTLIEB GMELIN,

MED. DOCT. ACADEM. IMPER. PETROPOL. BOTANICES PROFESSORE ET MEMBRO ORDINARIO

PETROPOLI

EX TYPOGRAPHIA ACADEMIAE SCIENTIARVM

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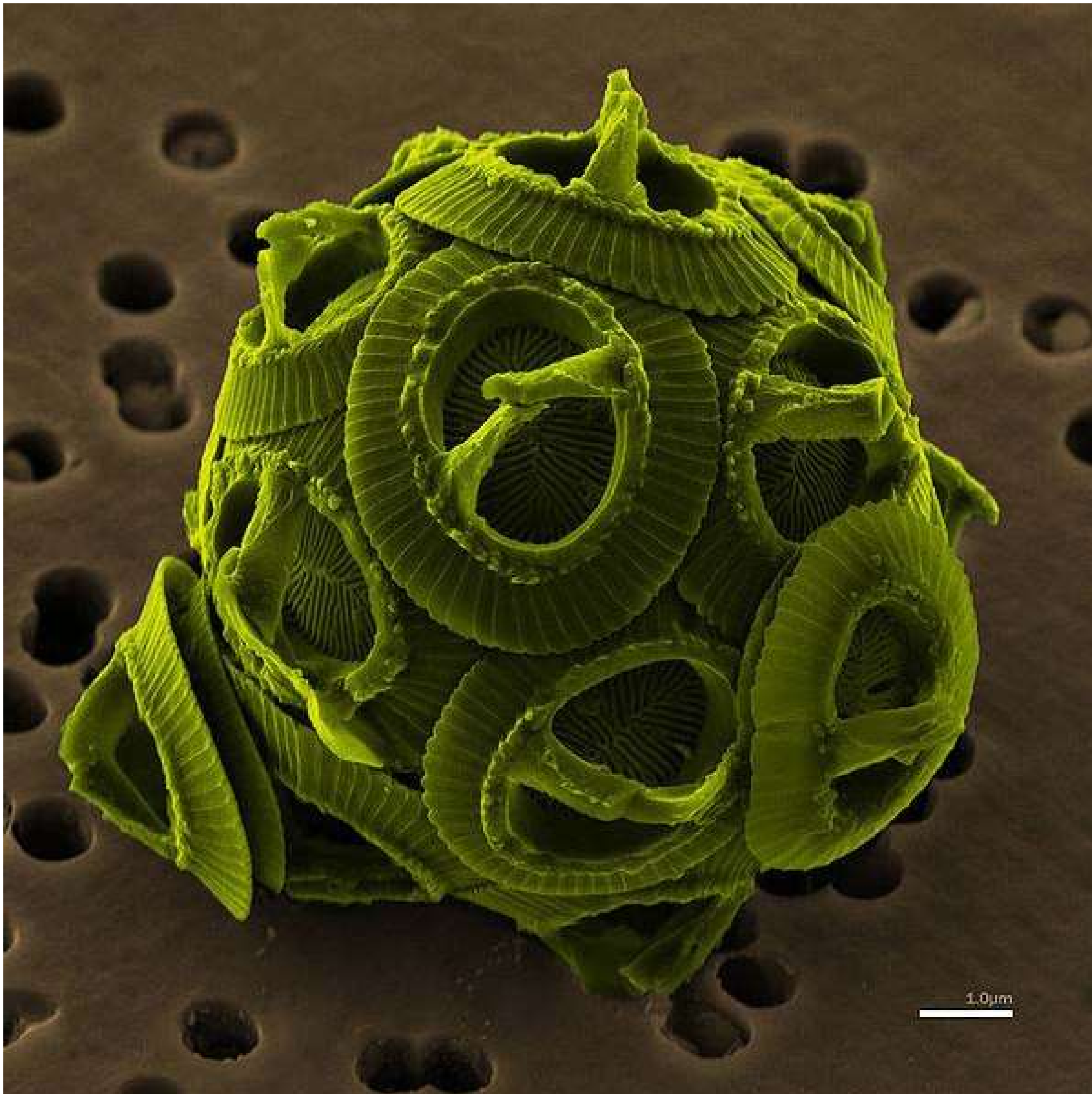
Title page of Samuel Gottlieb Gmelin, *Historia Fucorum*, dated 1768.

The singular *alga* is the Latin word for a particular seaweed and retains that meaning in English. The etymology is obscure. Although some speculate that it is related to Latin *algēre*, "be cold", there is no known reason to associate seaweed with temperature. A more likely source is *alliga*, "binding, entwining." Since Algae has become a biological classification, alga can also mean one classification under Algae, parallel to a fungus being a species of fungi, a plant being a species of plant, and so on.

The ancient Greek word for seaweed was *φῦκος* (fūkos or phykos), which could mean either the seaweed, probably Red Algae, or a red dye derived from it. The Latinization, *fūcus*, meant primarily the cosmetic rouge. The etymology is uncertain, but a strong candidate has long been some word related to the Biblical פוך (pūk), "paint" (if not that word itself), a cosmetic eye-shadow used by the ancient Egyptians and other inhabitants of the eastern Mediterranean. It could be any color: black, red, green, blue.

Accordingly the modern study of marine and freshwater algae is called either phycology or algology. The name Fucus appears in a number of taxa.The singular form is alga.

Classification



False-colour Scanning electron micrograph of the unicellular coccolithophore, *Gephyrocapsa oceanica*.

While *Cyanobacteria* have been traditionally included among the Algae, recent works usually exclude them due to large differences such as the lack of membrane-bound organelles, the presence of a single circular chromosome, the presence of peptidoglycan in the cell walls, and ribosomes different in size and content from those of the Eukaryotes. Rather than in chloroplasts, they conduct photosynthesis on specialized infolded cytoplasmic membranes called thylakoid membranes. Therefore, they differ significantly from the Algae despite occupying similar ecological niches.

By modern definitions Algae are Eukaryotes and conduct photosynthesis within membrane-bound organelles called chloroplasts. Chloroplasts contain circular DNA and are similar in structure to Cyanobacteria, presumably representing reduced cyanobacterial endosymbionts. The exact nature of the chloroplasts is different among the different lines of Algae, reflecting different endosymbiotic events. The table below describes the composition of the three major groups of Algae. Their lineage relationships are shown in the figure in the upper right. Many of these groups contain some members that are no longer photosynthetic. Some retain plastids, but not chloroplasts, while others have lost plastids entirely. The singular form is alga.

Supergroup affiliation	Members	Endosymbiont	Summary
Primoplantae/ Archaeplastida	• Chlorophyta • Rhodophyta • Glaucophyta	Cyanobacteria	These Algae have *primary* chloroplasts, i.e. the chloroplasts are surrounded by *two membranes* and probably developed through a single endosymbiotic event. The chloroplasts of Red Algae have chlorophylls *a* and *c* (often), and phycobilins, while those of Green Algae have chloroplasts with chlorophyll *a* and *b*. Higher plants are pigmented similarly to Green Algae and probably developed from them, and thus Chlorophyta is a sister taxon to the plants; sometimes they are grouped as Viridiplantae.
Excavata and Rhizaria	• Chlorarachniophytes • Euglenids	Green Algae	These groups have green chloroplasts containing chlorophylls *a* and *b*.

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			Their chloroplasts are surrounded by *four and three membranes*, respectively, and were probably retained from ingested Green Algae. **Chlorarachniophytes**, which belong to the phylum Cercozoa, contain a small nucleomorph, which is a relict of the algae's nucleus. **Euglenids**, which belong to the phylum Euglenozoa, live primarily in freshwater and have chloroplasts with only three membranes. It has been suggested that the endosymbiotic Green Algae were acquired through myzocytosis rather than phagocytosis.
Chromista and Alveolata	• Heterokonts • Haptophyta • Cryptomonads • Dinoflagellates	Red Algae	These groups have chloroplasts containing chlorophylls *a* and *c*, and phycobilins.The shape varies from plant to plant. they may be of discoid, plate-like, reticulate, cup-shaped, spiral or ribbon shaped. They have one or more pyrenoids to preserve protein and starch. The latter chlorophyll type is not known from any prokaryotes or primary chloroplasts, but genetic similarities with the Red Algae suggest a

relationship there.

In the first three of these groups (**Chromista**), the chloroplast has four membranes, retaining a nucleomorph in Cryptomonads, and they likely share a common pigmented ancestor, although other evidence casts doubt on whether the Heterokonts, Haptophyta, and Cryptomonads are in fact more closely related to each other than to other groups.

The typical **dinoflagellate** chloroplast has three membranes, but there is considerable diversity in chloroplasts within the group, and it appears there were a number of endosymbiotic events. The Apicomplexa, a group of closely related parasites, also have plastids called apicoplasts. Apicoplasts are not photosynthetic but appear to have a common origin with Dinoflagellate chloroplasts.

W.H.Harvey (1811—1866) was the first to divide the Algae into four divisions based on their pigmentation. This is the first use of a biochemical criterion in plant systematics. Harvey's four divisions are: Red Algae (Rhodophyta), Brown Algae (Heteromontophyta), Green Algae (Chlorophyta) and Diatomaceae.

Relationship to higher plants

The first plants on earth evolved from shallow freshwater algae much like *Chara* some 400 million years ago. These probably had an isomorphic alternation of generations and were probably filamentous. Fossils of isolated land plant spores suggest land plants may have been around as long as 475 million years ago.

Morphology

The kelp forest exhibit at the Monterey Bay Aquarium. A three-dimensional, multicellular thallus.

A range of algal morphologies are exhibited, and convergence of features in unrelated groups is common. The only groups to exhibit three dimensional multicellular thalli are the reds and browns, and some chlorophytes. Apical growth is constrained to subsets of these groups: the florideophyte reds, various browns, and the charophytes. The form of charophytes is quite different to those of reds and browns, because have distinct nodes, separated by internode 'stems'; whorls of branches reminiscent of the horsetails occur at the nodes. Conceptacles are another polyphyletic trait; they appear in the coralline algae and the Hildenbrandiales, as well as the browns.

Most of the simpler algae are unicellular flagellates or amoeboids, but colonial and non-motile forms have developed independently among several of the groups. Some of the more common organizational levels, more than one of which may occur in the life cycle of a species, are

- *Colonial*: small, regular groups of motile cells
- *Capsoid*: individual non-motile cells embedded in mucilage
- *Coccoid*: individual non-motile cells with cell walls
- *Palmelloid*: non-motile cells embedded in mucilage
- *Filamentous*: a string of non-motile cells connected together, sometimes branching
- *Parenchymatous*: cells forming a thallus with partial differentiation of tissues

In three lines even higher levels of organization have been reached, with full tissue differentiation. These are the brown algae,—some of which may reach 50 m in length (kelps)—the red algae, and the green algae. The most complex forms are found among the green algae, in a lineage that eventually led to the higher land plants. The point where these non-algal plants begin and algae stop is usually taken to be the presence of reproductive organs with protective cell layers, a characteristic not found in the other alga groups.

Symbiotic algae

Some species of algae form symbiotic relationships with other organisms. In these symbioses, the algae supply photosynthates (organic substances) to the host organism providing protection to the algal cells. The host organism derives some or all of its energy requirements from the algae. Examples are as follows.

Lichens

Rock lichens in Ireland.

Lichens are defined by the International Association for Lichenology to be "an association of a fungus and a photosynthetic symbiont resulting in a stable vegetative body having a specific structure." The fungi, or mycobionts, are from the Ascomycota with a few from the Basidiomycota. They are not found alone in nature but when they began to associate is not known. One mycobiont associates with the same phycobiont species, rarely two, from the Green Algae, except that alternatively the mycobiont may associate with the same species of Cyanobacteria (hence "photobiont" is the more accurate term). A photobiont may be associated with many specific mycobionts or live independently; accordingly, lichens are named and classified as fungal species. The association is termed a morphogenesis because the lichen has a form and capabilities not possessed by the symbiont species alone (they can be experimentally isolated). It is possible that the photobiont triggers otherwise latent genes in the mycobiont.

WORLD TECHNOLOGIES

Coral reefs

Floridian coral reef

Coral reefs are accumulated from the calcareous exoskeletons of marine invertebrates of the Scleractinia order; i.e., the Stony Corals. As animals they metabolize sugar and oxygen to obtain energy for their cell-building processes, including secretion of the exoskeleton, with water and carbon dioxide as byproducts. As the reef is the result of a favorable equilibrium between construction by the corals and destruction by marine erosion, the rate at which metabolism can proceed determines the growth or deterioration of the reef.

Algae of the Dinoflagellate phylum are often endosymbionts in the cells of marine invertebrates, where they accelerate host-cell metabolism by generating immediately available sugar and oxygen through photosynthesis using incident light and the carbon dioxide produced in the host. Endosymbiont algae in the Stony Corals are described by the term zooxanthellae, with the host Stony Corals called on that account hermatypic corals, which although not a taxon are not in healthy condition without their endosymbionts. Zooxanthellae belong almost entirely to the genus *Symbiodinium*. The loss of *Symbiodinium* from the host is known as coral bleaching, a condition which unless corrected leads to the deterioration and loss of the reef.

Sea sponges

Green Algae live close to the surface of some sponges, for example, breadcrumb sponge (*Halichondria panicea*). The alga is thus protected from predators; the sponge is provided with oxygen and sugars which can account for 50 to 80% of sponge growth in some species.

Life-cycle

Rhodophyta, Chlorophyta and Heterokontophyta, the three main algal Phyla, have life-cycles which show tremendous variation with considerable complexity. In general there is an asexual phase where the seaweed's cells are diploid, a sexual phase where the cells are haploid followed by fusion of the male and female gametes. Asexual reproduction is advantageous in that it permits efficient population increases, but less variation is possible. Sexual reproduction allows more variation, but is more costly. Often there is no strict alternation between the sporophyte and also because there is often an asexual phase, which could include the fragmentation of the thallus.

Numbers

Algae on coastal rocks at Shihtiping in Taiwan

The *Algal Collection of the U.S. National Herbarium* (located in the National Museum of Natural History) consists of approximately 320500 dried specimens, which, although not exhaustive (no exhaustive collection exists), gives an idea of the order of magnitude of the number of algal species (that number remains unknown). Estimates vary widely. For example, according to one standard textbook, in the British Isles the *UK Biodiversity Steering Group Report* estimated there to be 20000 algal species in the UK. Another checklist reports only about 5000 species. Regarding the difference of about 15000 species, the text concludes: "It will require many detailed field surveys before it is possible to provide a reliable estimate of the total number of species ...."

Regional and group estimates have been made as well: 5000—5500 species of Red Algae worldwide, "some 1300 in Australian Seas," 400 seaweed species for the western coastline of South Africa, 669 marine species from California (U.S.A.), 642 in the check-list of Britain and Ireland, and so on, but lacking any scientific basis or reliable sources, these numbers have no more credibility than the British ones mentioned above. Most estimates also omit the microscopic Algae, such as the phytoplankta, entirely.

Distribution

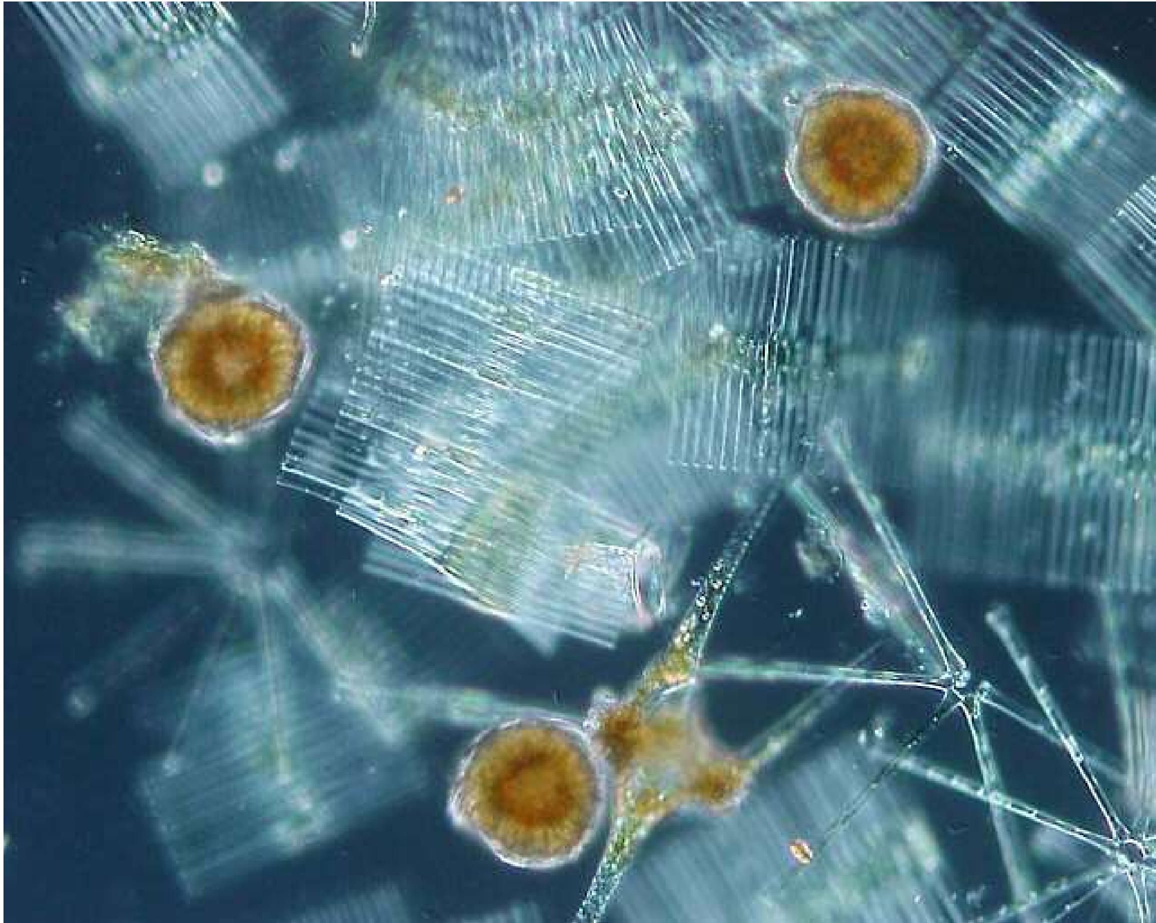
The topic of distribution of algal species has been fairly well studied since the founding of phytogeography in the mid-19th century AD. Algae spread mainly by the dispersal of spores analogously to the dispersal of Plantae by seeds and spores. Spores are everywhere in all parts of the Earth: the waters fresh and marine, the atmosphere, free-floating and in precipitation or mixed with dust, the humus and in other organisms, such as humans. Whether a spore is to grow into an organism depends on the combination of the species and the environmental conditions of where the spore lands.

The spores of fresh-water Algae are dispersed mainly by running water and wind, as well as by living carriers. The bodies of water into which they are transported are chemically selective. Marine spores are spread by currents. Ocean water is temperature selective, resulting in phytogeographic zones, regions and provinces.

To some degree the distribution of Algae is subject to floristic discontinuities caused by geographical features, such as Antarctica, long distances of ocean or general land masses. It is therefore possible to identify species occurring by locality, such as "Pacific Algae" or "North Sea Algae". When they occur out of their localities, it is usually possible to hypothesize a transport mechanism, such as the hulls of ships. For example, *Ulva reticulata* and *Ulva fasciata* travelled from the mainland to Hawaii in this manner.

Mapping is possible for select species only: "there are many valid examples of confined distribution patterns." For example, *Clathromorphum* is an arctic genus and is not mapped far south of there. On the other hand, scientists regard the overall data as insufficient due to the "difficulties of undertaking such studies."

Locations



Phytoplankton, Lake Chuzenji

Algae are prominent in bodies of water, common in terrestrial environments and are found in unusual environments, such as on snow and on ice. Seaweeds grow mostly in shallow marine waters, under 100 metres (330 ft); however some have been recorded to a depth of 360 metres (1,180 ft).

The various sorts of algae play significant roles in aquatic ecology. Microscopic forms that live suspended in the water column (phytoplankton) provide the food base for most marine food chains. In very high densities (algal blooms) these algae may discolor the water and outcompete, poison, or asphyxiate other life forms.

Algae are variously sensitive to different factors, which has made them useful as biological indicators in the Ballantine Scale and its modification.

Uses

Harvesting Algae

Agar

Agar, a gelatinous substance derived from red algae, has a number of commercial uses.

Alginates

Between 100,000 and 170,000 wet tons of *Macrocystis* are harvested annually in California for alginate extraction and abalone feed.

Energy source

To be competitive and independent from fluctuating support from (local) policy on the long run, biofuels should equal or beat the cost level of fossil fuels. Here, algae based fuels hold great promise, directly related to the potential to produce more biomass per unit area in a year than any other form of biomass. The break-even point for algae-based biofuels should be within reach in about ten to fifteen years.

Fertilizer

Seaweed is used as a fertilizer.

For centuries seaweed has been used as a fertilizer; George Owen of Henllys writing in the 16th century referring to drift weed in South Wales:

This kind of ore they often gather and lay on great heapes, where it heteth and rotteth, and will have a strong and loathsome smell; when being so rotten they cast on the land, as they do their muck, and thereof springeth good corn, especially barley ... After spring-tydes or great rigs of the sea, they fetch it in sacks on horse backes, and carie the same three, four, or five miles, and cast it on the lande, which doth very much better the ground for corn and grass.

Today Algae are used by humans in many ways; for example, as fertilizers, soil conditioners and livestock feed. Aquatic and microscopic species are cultured in clear tanks or ponds and are either harvested or used to treat effluents pumped through the ponds. Algaculture on a large scale is an important type of aquaculture in some places. Maerl is commonly used as a soil conditioner.

Nutrition

Seaweed gardens on Inisheer.

Naturally growing seaweeds are an important source of food, especially in Asia. They provide many vitamins including: A, B_1, B_2, B_6, niacin and C, and are rich in iodine, potassium, iron, magnesium and calcium. In addition commercially cultivated microalgae, including both Algae and Cyanobacteria, are marketed as nutritional supplements, such as Spirulina, Chlorella and the Vitamin-C supplement, Dunaliella, high in beta-carotene.

Algae are national foods of many nations: China consumes more than 70 species, including *fat choy*, a cyanobacterium considered a vegetable; Japan, over 20 species; Ireland, dulse; Chile, cochayuyo. Laver is used to make "laver bread" in Wales where it is known as *bara lawr*; in Korea, gim; in Japan, nori and aonori. It is also used along the west coast of North America from California to British Columbia, in Hawaii and by the Māori of New Zealand. Sea lettuce and badderlocks are a salad ingredient in Scotland, Ireland, Greenland and Iceland.

Dulse, a food.

The oils from some Algae have high levels of unsaturated fatty acids. For example, *Parietochloris incisa* is very high in arachidonic acid, where it reaches up to 47% of the triglyceride pool. Some varieties of Algae favored by vegetarianism and veganism contain the long-chain, essential omega-3 fatty acids, Docosahexaenoic acid (DHA) and Eicosapentaenoic acid (EPA), in addition to vitamin B_{12}. The vitamin B_{12} in algae is not biologically active. Fish oil contains the omega-3 fatty acids, but the original source is algae (microalgae in particular), which are eaten by marine life such as copepods and are passed up the food chain. Algae has emerged in recent years as a popular source of omega-3 fatty acids for vegetarians who cannot get long-chain EPA and DHA from other vegetarian sources such as flaxseed oil, which only contains the short-chain Alpha-Linolenic acid (ALA).

Pollution control

- Sewage can be treated with algae, reducing the need for greater amounts of toxic chemicals than are already used.
- Algae can be used to capture fertilizers in runoff from farms. When subsequently harvested, the enriched algae itself can be used as fertilizer.

Agricultural Research Service scientists found that 60-90% of nitrogen runoff and 70-100% of phosphorus runoff can be captured from manure effluents using an algal turf scrubber (ATS). Scientists developed the ATS, which are shallow, 100-foot raceways of nylon netting where algae colonies can form, and studied its efficacy for three years. They found that algae can readily be used to reduce the nutrient runoff from agricultural fields and increase the quality of water flowing into rivers, streams, and oceans. The enriched algae itself also can be used as a fertilizer. Researchers collected and dried the nutrient-rich algae from the ATS and studied its potential as an organic fertilizer. They found that cucumber and corn seedlings grew just as well using ATS organic fertilizer as they did with commercial fertilizers.

Pigments

The natural pigments produced by algae can be used as an alternative to chemical dyes and coloring agents.

Stabilizing substances

Carrageenan, from the red alga *Chondrus crispus*, is used as a stabiliser in milk products.

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Chapter 3

Industrial Microbiology

Industrial microbiology or microbial biotechnology encompasses the use of microorganisms in the manufacture of food or industrial products. The use of microorganisms for the production of food, either human or animal, is often considered a branch of food microbiology. The microorganisms used in industrial processes may be natural isolates, laboratory selected mutants or genetically engineered organisms.

Food microbiology

Yogurt, cheese, chocolate, and silage (animal food) are all produced by industrial microbiology processes. 'Good' bacteria such as probiotics are becoming increasingly important in the food industry. Lactic Acid Bacteria and Bifidobacteria are amongst the most important groups of microorganisms used in the food industry. These bacteria are thought to have health-promoting abilities and many are used as probiotics for the prevention, alleviation and treatment of intestinal disorders in humans and animals.

Biopolymers

A huge variety of biopolymers, such as polysaccharides, polyesters, and polyamides, are produced by microorganisms. These products range from viscous solutions to plastics. The genetic manipulation of microorganisms has permitted the biotechnological production of biopolymers with tailored material properties suitable for high-value medical application such as tissue engineering and drug delivery. Industrial microbiology can be used for the biosynthesis of xanthan, alginate, cellulose, cyanophycin, poly(gamma-glutamic acid), levan, hyaluronic acid, organic acids, oligosaccharides and polysaccharides, and polyhydroxyalkanoates.

Bioremediation

Microbial biodegradation of pollutants can be used to cleanup contaminated environments. These bioremediation and biotransformation methods harness naturally occurring microbes to degrade, transform or accumulate a huge range of compounds including hydrocarbons (e.g. oil), polychlorinated biphenyls (PCBs), polyaromatic hydrocarbons (PAHs), pharmaceutical substances, radionuclides and metals.

Waste biotreatment

Microorganisms are used to treat the vast quantities of wastes generated by modern societies. Biotreatment, the processing of wastes using living organisms, is an environmentally friendly, relatively simple and cost-effective alternative to physico-chemical clean-up options. Confined environments, such as bioreactors can be employed in biotreatment processes.

Wastewater treatment

Biological wastewater treatment is undoubtedly one of the most important biotechnological processes, which have been used for over a century to treat municipal and industrial wastewaters. Culture-independent molecular techniques have been used to study the diversity and physiology of ecologically important microorganisms in wastewater treatment processes. A number of new exciting insights into the structure, function, and dynamics of complex microbial communities in wastewater treatment processes have been gained, which have significantly expanded our understanding of process design, operation and control. Microbes play a vital role in the cycling of nitrogen in wastewater treatment processes (including anaerobic ammonia oxidation processes) and methane fermentation processes.

Health-care and medicine

Microorganisms are used to produce human or animal biologicals such as insulin, growth hormone, and antibodies. Diagnostic assays that use monoclonal antibody, DNA probe technology or real-time PCR are used as rapid tests for pathogenic organisms in the clinical laborarory.

Microorganisms may also help in the treatment of diseases such as cancer. Research shows that clostridia can selectively target cancer cells. Various strains of non-pathogenic clostridia have been shown to infiltrate and replicate within solid tumours. Clostridia therefore have the potential to deliver therapeutic proteins to tumours. *Lactobacillus* spp. and other lactic acid bacteria possess numerous potential therapeutic properties including anti-inflammatory and anti-cancer activities.

Vaccines are used to combat infectious disease, however the last decade has witnessed a revolution in the approach to vaccine design and development. Sophisticated

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technologies such as genomics, proteomics, functional genomics, and synthetic chemistry can be used for the rational identification of antigens, the synthesis of complex glycans, and the generation of engineered carrier proteins.

Members of the *Streptomyces* genus are among the most prolific microorganisms producing secondary metabolites with wide uses in medicine and in agriculture. These organisms have a complex secondary metabolism producing antibiotic compounds and other metabolites with medicinal properties. Genomic studies, genomic mining and biotechnological approaches are being employed in the search for new antibiotics and other drugs in *Streptomyces*.

Archaea

Examination of microbes living in unusual environments (e.g. high temperatures, salt, low pH or temperature, high radiation) lead to discovery of microbes with new abilities that can be harnessed for industrial purposes.

Corynebacteria

Corynebacteria are a diverse group Gram-positive bacteria found in a range of different ecological niches such as soil, vegetables, sewage, skin, and cheese smear. *Corynebacterium glutamicum* is of immense industrial importance and is one of the biotechnologically most important bacterial species with an annual production of more than two million tons of amino acids, mainly L-glutamate and L-lysine. The genome sequence of *C. glutamicum* has been published.

Xanthomonas

The genus *Xanthomonas* consists of 20 plant-associated species, many of which cause important diseases of crops and other plants. Individual species comprise multiple pathovars, characterized by distinctive host specificity or mode of infection. Bacteria of the genus *Xanthomonas* are able to produce the acidic exopolysaccharide xanthan gum. Because of its physical properties, it is widely used as a viscosifer, thickener, emulsifier or stabilizer in both food and non-food industries.

Aspergillus

Species within the genus *Aspergillus* have a large chemical repertoire. Commodity products produced in *Aspergillus* cell 'factories' include citric, gluconic, itaconic and kojic acid. The use of *Aspergillus niger* in citric acid production dates back to 1917. Citric acid is one of the most widely used food ingredients. It also has found use in the pharmaceutical and cosmetic industries as an acidulant and for aiding in the dissolution of active ingredients. Other technical applications of citric acid are as a hardener in adhesive and for retarding the setting of concrete. Citric acid is a true 'bulk chemical' with

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an estimated production approximating more than 1.6 billion kg each year *A. niger* also has found use in the industrial production of gluconic acid, which is used as an additive in certain metal cleaning applications, as well as for the therapy for calcium and iron deficiencies. *Aspergillus terreus* is used for itaconic acid production, a synthetic polymer. *A. oryzae* is fermented for kojic acid production which is used for skin whitening and as a precursor for synthesis of flavour enhancers.

Viruses

Viruses that are pathogenic to insect pests can be exploited as biological control agents. Some viruses such as baculoviruses have been exploited for use as gene expression and delivery vectors in both insect and mammalian cells.

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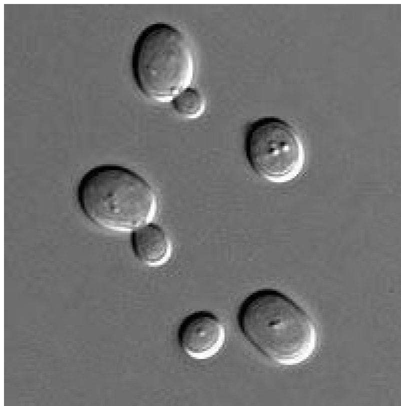
Chapter 4

Yeast

Yeast	
 Yeast of the species *Saccharomyces cerevisiae*	
Scientific classification	
Domain:	Eukaryota
Kingdom:	Fungi
Phyla and Subphyla	

Ascomycota

- Saccharomycotina (true yeasts)
- Taphrinomycotina
  - Schizosaccharomycetes (fission yeasts)

Basidiomycota

- Agaricomycotina
  - Tremellomycetes
- Pucciniomycotina
  - Microbotryomycetes

Yeasts are eukaryotic micro-organisms classified in the kingdom Fungi, with the 1,500 species currently described estimated to be only 1% of all yeast species. Most reproduce asexually by budding, although a few do so by mitosis. Yeasts are unicellular, although some species with yeast forms may become multicellular through the formation of a string of connected budding cells known as pseudohyphae, or false hyphae, as seen in most molds. Yeast size can vary greatly depending on the species, typically measuring 3–4 µm in diameter, although some yeasts can reach over 40 µm.

The yeast species *Saccharomyces cerevisiae* has been used in baking and in fermenting alcoholic beverages for thousands of years. It is also extremely important as a model organism in modern cell biology research, and is one of the most thoroughly researched eukaryotic microorganisms. Researchers have used it to gather information about the biology of the eukaryotic cell and ultimately human biology. Other species of yeast, such as *Candida albicans*, are opportunistic pathogens and can cause infections in humans. Yeasts have recently been used to generate electricity in microbial fuel cells, and produce ethanol for the biofuel industry.

Yeasts do not form a single taxonomic or phylogenetic grouping. The term "yeast" is often taken as a synonym for *Saccharomyces cerevisiae*, but the phylogenetic diversity of yeasts is shown by their placement in two separate phyla, the Ascomycota and the Basidiomycota. The budding yeasts ("true yeasts") are classified in the order Saccharomycetales.

History

The word "yeast" comes to us from Old English *gist*, *gyst*, and from the Indo-European root *yes-*, meaning *boil*, *foam*, or *bubble*. Yeast microbes are probably one of the earliest domesticated organisms. People have used yeast for fermentation and baking throughout history. Archaeologists digging in Egyptian ruins found early grinding stones and baking chambers for yeasted bread, as well as drawings of 4,000-year-old bakeries and breweries. In 1680, the Dutch naturalist Anton van Leeuwenhoek first microscopically observed yeast, but at the time did not consider them to be living organisms, but rather globular structures. In 1857, French microbiologist Louis Pasteur proved in the paper "*Mémoire sur la fermentation alcoolique*" that alcoholic fermentation was conducted by living yeasts and not by a chemical catalyst. Pasteur showed that by bubbling oxygen into the yeast broth, cell growth could be increased, but fermentation was inhibited – an observation later called the "Pasteur effect".

By the late 18th century, two yeast strains used in brewing had been identified: *Saccharomyces cerevisiae*, so called top fermenting yeast, and *S. carlsbergensis*, bottom fermenting yeast. *S. cerevisiae* has been sold commercially by the Dutch for bread making since 1780; while around 1800, the Germans started producing *S. cerevisiae* in the form of cream. In 1825 a method was developed to remove the liquid so the yeast could be prepared as solid blocks. The industrial production of yeast blocks was enhanced by the introduction of the filter press in 1867. In 1872, Baron Max de Springer developed a manufacturing process to create granulated yeast, a technique that was used

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until the first World War. In the United States, naturally occurring airborne yeasts were used almost exclusively until commercial yeast was marketed at the Centennial Exposition in 1876 in Philadelphia, where Charles L. Fleischmann exhibited the product and a process to use it, as well as serving the resultant baked bread.

Nutrition and growth

Yeasts are chemoorganotrophs, as they use organic compounds as a source of energy and do not require sunlight to grow. Carbon is obtained mostly from hexose sugars, such as glucose and fructose, or disaccharides such as sucrose and maltose. Some species can metabolize pentose sugars like ribose, alcohols, and organic acids. Yeast species either require oxygen for aerobic cellular respiration (obligate aerobes), or are anaerobic, but also have aerobic methods of energy production (facultative anaerobes). Unlike bacteria, there are no known yeast species that grow only anaerobically (obligate anaerobes). Yeasts grow best in a neutral or slightly acidic pH environment.

Yeasts vary in what temperature range they grow best. For example, *Leucosporidium frigidum* grows at -2 to 20 °C (28 to 68 °F), *Saccharomyces telluris* at 5 to 35 °C (41 to 95 °F) and *Candida slooffi* at 28 to 45 °C (82 to 113 °F). The cells can survive freezing under certain conditions, with viability decreasing over time.

Yeasts are generally grown in the laboratory on solid growth media or in liquid broths. Common media used for the cultivation of yeasts include potato dextrose agar (PDA) or potato dextrose broth, Wallerstein Laboratories nutrient (WLN) agar, yeast peptone dextrose agar (YPD), and yeast mould agar or broth (YM). Home brewers who cultivate yeast frequently use dried malt extract (DME) and agar as a solid growth medium. The antibiotic cycloheximide is sometimes added to yeast growth media to inhibit the growth of *Saccharomyces* yeasts and select for wild/indigenous yeast species. This will change the yeast process.

The appearance of a white, thready yeast, commonly known as kahm yeast, is often a byproduct of the lactofermentation (or pickling) of certain vegetables, usually the result of exposure to air. Although harmless, it can give pickled vegetables a bad flavour and so must be removed regularly during fermentation.

Ecology

Yeasts are very common in the environment, but are usually isolated from sugar-rich material. Examples include naturally occurring yeasts on the skins of fruits and berries (such as grapes, apples or peaches), and exudates from plants (such as plant saps or cacti). Some yeasts are found in association with soil and insects. The ecological function and biodiversity of yeasts are relatively unknown compared to those of other microorganisms. Yeasts, including *Candida albicans*, *Rhodotorula rubra*, *Torulopsis* and *Trichosporon cutaneum*, have been found living in between people's toes as part of their

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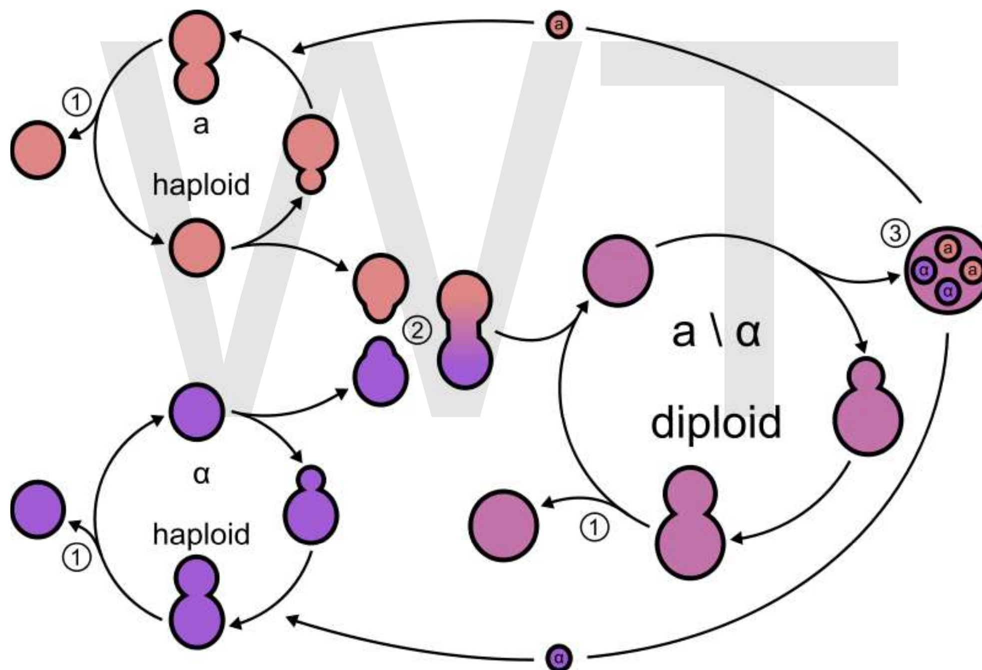
skin flora. Yeasts are also present in the gut flora of mammals and some insects and even deep-sea environments host an array of yeasts.

An Indian study of seven bee species and 9 plant species found 45 species from 16 genera colonise the nectaries of flowers and honey stomachs of bees. Most were members of the *Candida* genus; the most common species in honey stomachs was *Dekkera intermedia* and in flower nectaries, *Candida blankii*. Yeast colonising nectaries of the stinking hellebore have been found to raise the temperature of the flower, which may aid in attracting pollinators by increasing the evaporation of volatile organic compounds. A black yeast has been recorded as a partner in a complex relationship between ants, their mutualistic fungus, a fungal parasite of the fungus and a bacterium that kills the parasite. The yeast have a negative effect on the bacteria that normally produce antibiotics to kill the parasite and so may affect the ants' health by allowing the parasite to spread.

Reproduction



The yeast cell's life cycle:
1. Budding
2. Conjugation
3. Spore

Yeasts have asexual and sexual reproductive cycles. The most common mode of vegetative growth in yeast is asexual reproduction by budding. Here a small bud, or daughter cell, is formed on the parent cell. The nucleus of the parent cell splits into a daughter nucleus and migrates into the daughter cell. The bud continues to grow until it separates from the parent cell, forming a new cell. Some yeasts, including *Schizosaccharomyces pombe*, reproduce by mitosis instead of budding.

Under high stress conditions, haploid cells will generally die; under the same conditions, however, diploid cells can undergo sporulation, entering sexual reproduction (meiosis) and producing a variety of haploid spores, which can go on to mate (conjugate), reforming the diploid.

Uses

The useful physiological properties of yeast have led to their use in the field of biotechnology. Fermentation of sugars by yeast is the oldest and largest application of this technology. Many types of yeasts are used for making many foods: baker's yeast in bread production; brewer's yeast in beer fermentation; yeast in wine fermentation and for xylitol production. So-called red rice yeast is actually a mold, *Monascus purpureus*. Yeasts include some of the most widely used model organisms for genetics and cell biology.

Alcoholic beverages

Alcoholic beverages are defined as beverages that contain ethanol (C_2H_5OH). This ethanol is almost always produced by fermentation – the metabolism of carbohydrates by certain species of yeast under anaerobic or low-oxygen conditions. Beverages such as wine, beer, or distilled spirits all use yeast at some stage of their production. A distilled beverage is a beverage containing ethanol that has been purified by distillation. Carbohydrate-containing plant material is fermented by yeast, producing a dilute solution of ethanol in the process. Spirits such as whiskey and rum are prepared by distilling these dilute solutions of ethanol. Components other than ethanol are collected in the condensate, including water, esters, and other alcohols, which account for the flavour of the beverage.

Beer

Beer being fermented by brewers yeast

Brewing yeasts may be classed as "top cropping" (or "top fermenting") and "bottom cropping" (or "bottom-fermenting"). Top cropping yeasts are so called because they form a foam at the top of the wort during fermentation. An example of a top cropping yeast is *Saccharomyces cerevisiae*, sometimes called an "ale yeast". Bottom cropping yeasts are typically used to produce lager-type beers, though they can also produce ale-type beers. These yeasts ferment more sugars, creating a dryer beer, and grow well at low temperatures. An example of bottom cropping yeast is *Saccharomyces pastorianus*, formerly known as *S. carlsbergensis*.

The most common top cropping brewer's yeast, *S. cerevisiae*, is the same species as the common baking yeast. However, baking and brewing yeasts typically belong to different strains, cultivated to favour different characteristics: baking yeast strains are more aggressive, to carbonate dough in the shortest amount of time possible; brewing yeast strains act slower, but tend to produce fewer off-flavours and tolerate higher alcohol concentrations (with some strains, up to 22%).

Brettanomyces is a genus of wild yeast important in brewing lambic, a beer produced not by the deliberate addition of brewer's yeasts, but by spontaneous fermentation by wild yeasts and bacteria. *Brettanomyces lambicus*, *B. bruxellensis* and *B. claussenii* are native to the Senne Valley region of Belgium, where lambic beer is produced.

Wine

Fresh grapes with visible bloom.

Yeast is used in winemaking, where it converts the sugars present in grape juice (must) into ethanol. Yeast is normally already present on grape skins (the white powder called "the bloom"). Fermentation can be done with this endogenous "wild yeast," but this procedure gives unpredictable results, which depend upon the exact types of yeast species present. For this reason, a pure yeast culture is usually added to the must; this yeast quickly dominates the fermentation. The wild yeasts are repressed, which ensures a reliable and predictable fermentation.

Most added wine yeasts are strains of *S. cerevisiae*, though not all strains of the species are suitable. Different *S. cerevisiae* yeast strains have differing physiological and fermentative properties, therefore the actual strain of yeast selected can have a direct impact on the finished wine. Significant research has been undertaken into the development of novel wine yeast strains that produce atypical flavour profiles or increased complexity in wines.

The growth of some yeasts, such as *Zygosaccharomyces* and *Brettanomyces*, in wine can result in wine faults and subsequent spoilage. *Brettanomyces* produces an array of metabolites when growing in wine, some of which are volatile phenolic compounds.

Together, these compounds are often referred to as "*Brettanomyces* character", and are often described as "antiseptic" or "barnyard" type aromas. *Brettanomyces* is a significant contributor to wine faults within the wine industry.

Researchers from University of British Columbia, Canada, have found a new strain of yeast that has reduced amines. The amines in red wine and Chardonnay produce off-flavors and cause headaches and hypertension in some people. About 30 percent of people are sensitive to biogenic amines, such as histamines.

Baking

Yeast, most commonly *S. cerevisiae*, is used in baking as a leavening agent, where it converts the fermentable sugars present in dough into the gas carbon dioxide. This causes the dough to expand or rise as gas forms pockets or bubbles. When the dough is baked, the yeast dies and the air pockets "set", giving the baked product a soft and spongy texture. The use of potatoes, water from potato boiling, eggs, or sugar in a bread dough accelerates the growth of yeasts. Most yeasts used in baking are of the same species common in alcoholic fermentation. Additionally, *Saccharomyces exiguus* (also known as *S. minor*), a wild yeast found on plants, fruits, and grains, is occasionally used for baking. Sugar and vinegar provide the best conditions for yeast to ferment. In bread making, the yeast initially respires aerobically, producing carbon dioxide and water. When the oxygen is depleted, anaerobic respiration begins, producing ethanol as a waste product; however, this evaporates during baking.

A block of fresh yeast

It is not known when yeast was first used to bake bread. The first records that show this use came from Ancient Egypt. Researchers speculate a mixture of flour meal and water was left longer than usual on a warm day and the yeasts that occur in natural contaminants of the flour caused it to ferment before baking. The resulting bread would have been lighter and tastier than the normal flat, hard cake.

Active dried yeast, a granulated form in which yeast is commercially sold

Today, there are several retailers of baker's yeast; one of the best-known in North America is Fleischmann’s Yeast, which was developed in 1868. During World War II, Fleischmann's developed a granulated active dry yeast, which did not require refrigeration and had a longer shelf life than fresh yeast. The company created yeast that would rise twice as fast, reducing baking time. Baker's yeast is also sold as a fresh yeast compressed into a square "cake". This form perishes quickly, and must therefore be used soon after production. A weak solution of water and sugar can be used to determine if yeast is expired. In the solution, active yeast will foam and bubble as it ferments the sugar into ethanol and carbon dioxide. Some recipes refer to this as proofing the yeast as it "proves" (tests) the viability of the yeast before the other ingredients are added. When using a sourdough starter, flour and water are added instead of sugar; this is referred to as proofing the sponge.

When yeast is used for making bread, it is mixed with flour, salt, and warm water or milk. The dough is kneaded until it is smooth, and then left to rise, sometimes until it has doubled in size. Some bread doughs are knocked back after one rising and left to rise again. A longer rising time gives a better flavour, but the yeast can fail to raise the bread in the final stages if it is left for too long initially. The dough is then shaped into loaves, left to rise until it is the correct size, and then baked. Dried yeast is usually specified for use in a bread machine, however a (wet) sourdough starter can also work.

Bioremediation

Some yeasts can find potential application in the field of bioremediation. One such yeast, *Yarrowia lipolytica*, is known to degrade palm oil mill effluent, TNT (an explosive material), and other hydrocarbons, such as alkanes, fatty acids, fats and oils. It can also tolerate high concentrations of salt and heavy metals, and is being investigated for its potential as a heavy metal biosorbent.

Industrial ethanol production

The ability of yeast to convert sugar into ethanol has been harnessed by the biotechnology industry to produce ethanol fuel. The process starts by milling a feedstock, such as sugar cane, field corn, or other cereal grains, and then adding dilute sulfuric acid, or fungal alpha amylase enzymes, to break down the starches into complex sugars. A glucoamylase is then added to break the complex sugars down into simple sugars. After this, yeasts are added to convert the simple sugars to ethanol, which is then distilled off to obtain ethanol up to 96% in concentration.

Saccharomyces yeasts have been genetically engineered to ferment xylose, one of the major fermentable sugars present in cellulosic biomasses, such as agriculture residues, paper wastes, and wood chips. Such a development means ethanol can be efficiently produced from more inexpensive feedstocks, making cellulosic ethanol fuel a more competitively priced alternative to gasoline fuels.

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Nonalcoholic beverages

A Kombucha culture fermenting in a jar

Root beer and other sweet carbonated beverages can be produced using the same methods as beer, except the fermentation is stopped sooner, producing carbon dioxide, but only trace amounts of alcohol, and a significant amount of sugar is left in the drink. *Kvass*, a fermented drink made from rye, is popular in Eastern Europe; it has a recognizable, but low alcoholic content. Yeast in symbiosis with acetic acid bacteria is used in the preparation of *kombucha*, a fermented sweetened tea. Species of yeast found in the tea can vary, and may include: *Brettanomyces bruxellensis*, *Candida stellata*, *Schizosaccharomyces pombe*, *Torulaspora delbrueckii* and *Zygosaccharomyces bailii*. *Kombucha* is a popular beverage in Eastern Europe and some former Soviet republics

under the name *chajnyj grib* (Чайный гриб). *Kefir* and *kumis* are made by fermenting milk with yeast and bacteria.

Nutritional supplements

Yeast is used in nutritional supplements popular with vegans and the health conscious, where it is often referred to as "nutritional yeast". It is a deactivated yeast, usually *S. cerevisiae*. It is an excellent source of protein and vitamins, especially the B-complex vitamins, whose functions are related to metabolism, as well as other minerals and cofactors required for growth. It is also naturally low in fat and sodium. Some brands of nutritional yeast, though not all, are fortified with vitamin B_{12}, which is produced separately by bacteria. Nutritional yeast, though it has a similar appearance to brewer's yeast, is very different and has a very different taste.

Nutritional yeast has a nutty, cheesy, creamy flavor which makes it popular as an ingredient in cheese substitutes. It is often used by vegans in place of Parmesan cheese. Another popular use is as a topping for popcorn. It can also be used in mashed and fried potatoes, as well as in scrambled eggs. It comes in the form of flakes, or as a yellow powder similar in texture to cornmeal, and can be found in the bulk aisle of most natural food stores. In Australia, it is sometimes sold as "savory yeast flakes". Though "nutritional yeast" usually refers to commercial products, inadequately fed prisoners have used "home-grown" yeast to prevent vitamin deficiency.

Probiotics

Some probiotic supplements use the yeast *S. boulardii* to maintain and restore the natural flora in the gastrointestinal tract. *S. boulardii* has been shown to reduce the symptoms of acute diarrhea in children, prevent reinfection of *Clostridium difficile*, reduce bowel movements in diarrhea-predominant IBS patients, and reduce the incidence of antibiotic, traveler's, and HIV/AIDS associated diarrheas.

Aquarium hobby

Yeast is often used by aquarium hobbyists to generate carbon dioxide (CO_2) to nourish plants in planted aquariums. A homemade setup is widely used as a cheap and simple alternative to pressurized CO_2 systems. While not as effective as these, the homemade setup is considerably cheaper for less demanding hobbyists.

There are several recipes for homemade CO_2, but they are variations of the basic recipe: Baker's yeast, with sugar, baking soda and water, are added to a plastic bottle. A few drops of vegetable oil at the start reduces surface tension and speeds the release of CO_2. This will produce CO_2 for about 2 or 3 weeks; the use of a bubble counter determines production. The CO_2 is injected in the aquarium via a narrow hose and released through a diffuser that helps dissolve the gas in the water. The CO_2 is used by plants in the photosynthesis process.

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Science

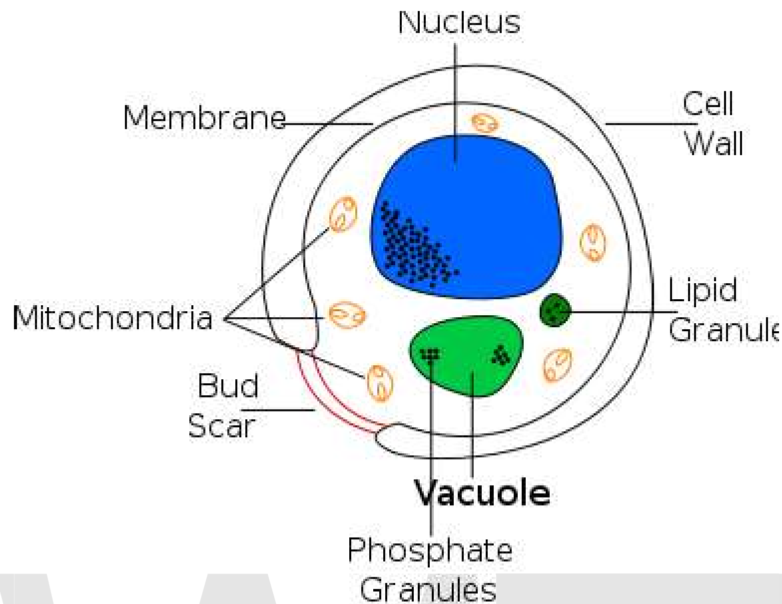


Diagram showing a yeast cell

Several yeasts, particularly *S. cerevisiae*, have been widely used in genetics and cell biology. This is largely because *S. cerevisiae* is a simple eukaryotic cell, serving as a model for all eukaryotes, including humans for the study of fundamental cellular processes such as the cell cycle, DNA replication, recombination, cell division and metabolism. Also, yeasts are easily manipulated and cultured in the laboratory, which has allowed for the development of powerful standard techniques, such as yeast two-hybrid, synthetic genetic array analysis and tetrad analysis. Many proteins important in human biology were first discovered by studying their homologues in yeast; these proteins include cell cycle proteins, signaling proteins, and protein-processing enzymes.

On 24 April 1996 *S. cerevisiae* was announced to be the first eukaryote to have its genome, consisting of 12 million base pairs, fully sequenced as part of the Genome project. At the time, it was the most complex organism to have its full genome sequenced, and took seven years and the involvement of more than 100 laboratories to accomplish. The second yeast species to have its genome sequenced was *Schizosaccharomyces pombe*, which was completed in 2002. It was the sixth eukaryotic genome sequenced and consists of 13.8 million base pairs.

Yeast extract

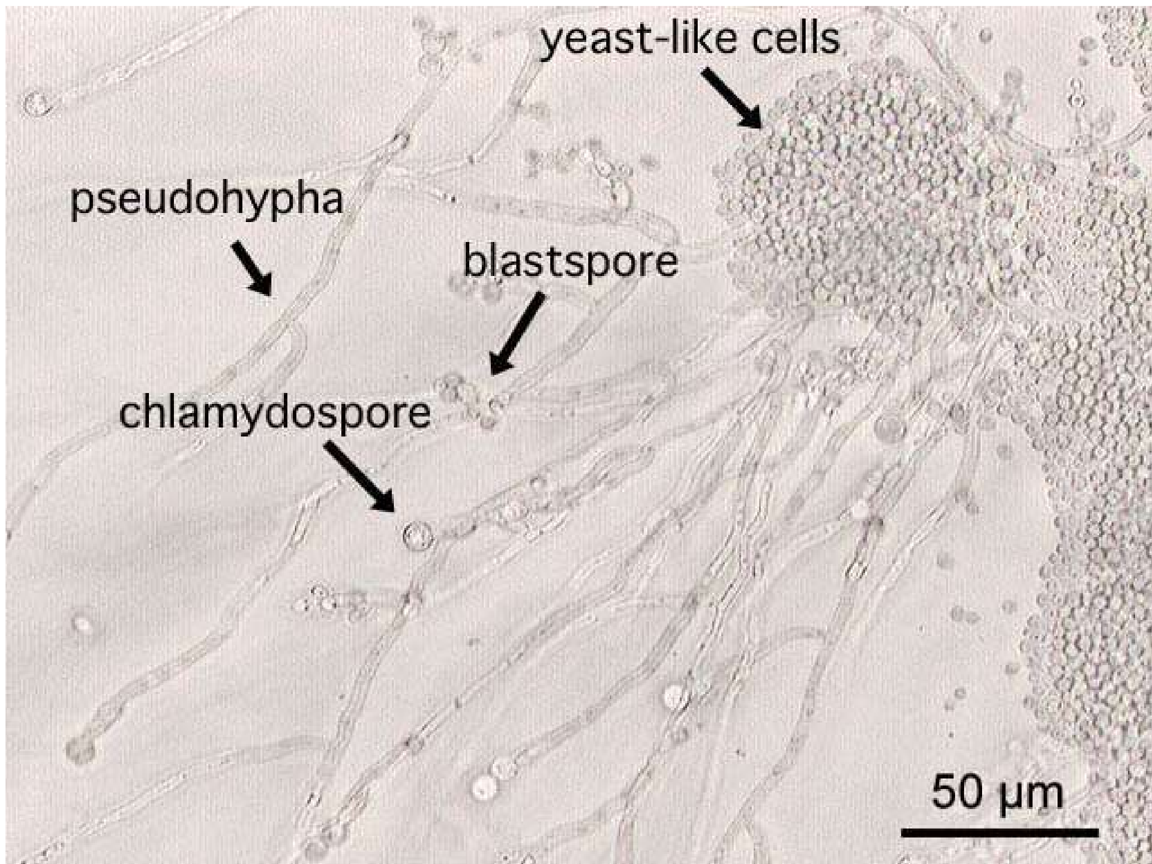
Marmite and Vegemite have a distinctive dark colour

Marmite and Vegemite, products made from yeast extract

Yeast extract is the common name for various forms of processed yeast products that are used as food additives or flavours. They are often used in the same way that monosodium glutamate (MSG) is used, and like MSG, often contain free glutamic acid. The general method for making yeast extract for food products such as Vegemite and Marmite on a commercial scale is to add salt to a suspension of yeast making the solution hypertonic, which leads to the cells shrivelling up. This triggers *autolysis*, where the yeast's digestive enzymes break their own proteins down into simpler compounds, a process of self-

destruction. The dying yeast cells are then heated to complete their breakdown, after which the husks (yeast with thick cell walls which would give poor texture) are separated. Yeast autolysates are used in Vegemite and Promite (Australia); Marmite, Bovril and Oxo (the United Kingdom, Republic of Ireland and South Africa); and Cenovis (Switzerland).

Pathogenic yeasts



A photomicrograph of *Candida albicans* showing hyphal outgrowth and other morphological characteristics.

Some species of yeast are opportunistic pathogens where they can cause infection in people with compromised immune systems.

Cryptococcus neoformans is a significant pathogen of immunocompromised people causing the disease termed cryptococcosis. This disease occurs in about 7–9% of AIDS patients in the USA, and a slightly smaller percentage (3–6%) in western Europe. The cells of the yeast are surrounded by a rigid polysaccharide capsule, which helps to prevent them from being recognised and engulfed by white blood cells in the human body.

Yeasts of the *Candida* genus are another group of opportunistic pathogens which causes oral and vaginal infections in humans, known as candidiasis. *Candida* is commonly found as a commensal yeast in the mucus membranes of humans and other warm-blooded animals. However, sometimes these same strains can become pathogenic. Here the yeast cells sprout a hyphal outgrowth, which locally penetrates the mucosal membrane, causing irritation and shedding of the tissues. The pathogenic yeasts of candidiasis in probable descending order of virulence for humans are: *C. albicans*, *C. tropicalis*, *C. stellatoidea*, *C. glabrata*, *C. krusei*, *C. parapsilosis*, *C. guilliermondii*, *C. viswanathii*, *C. lusitaniae* and *Rhodotorula mucilaginosa*. *Candida glabrata* is the second most common *Candida* pathogen after *C. albicans*, causing infections of the urogenital tract, and of the bloodstream (candidemia).

Food spoilage

Yeasts are able to grow in foods with a low pH, (5.0 or lower) and in the presence of sugars, organic acids and other easily metabolized carbon sources. During their growth, yeasts metabolize some food components and produce metabolic end products. This causes the physical, chemical, and sensible properties of a food to change, and the food is spoiled. The growth of yeast within food products is often seen on their surface, as in cheeses or meats, or by the fermentation of sugars in beverages, such as juices, and semi-liquid products, such as syrups and jams. The yeast of the *Zygosaccharomyces* genus have had a long history as a spoilage yeast within the food industry. This is mainly due to the fact that these species can grow in the presence of high sucrose, ethanol, acetic acid, sorbic acid, benzoic acid, and sulfur dioxide concentrations, representing some of the commonly used food preservation methods. Methylene blue is used to test for the presence of live yeast cells.

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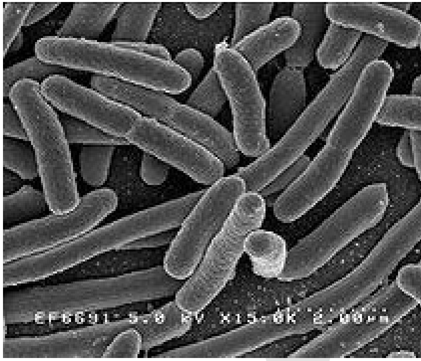
Chapter 5

Bacteria

Bacteria Temporal range: Archean or earlier – Recent	
 Scanning electron micrograph of *Escherichia coli* bacilli	
Scientific classification	
Domain:	**Bacteria**
Phyla	

- **gram positive/no outer membrane**

Actinobacteria (high-G+C)
Firmicutes (low-G+C)
Tenericutes (no wall)

- **gram negative/outer membrane present**

Aquificae
Deinococcus-Thermus
Fibrobacteres–

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Chlorobi/Bacteroidetes
(FCB group)
Fusobacteria
Gemmatimonadetes
Nitrospirae
Planctomycetes–
Verrucomicrobia/Chlamydiae
(PVC group)
Proteobacteria
Spirochaetes
Synergistetes

- **unknown/ungrouped**

Acidobacteria
Chloroflexi
Chrysiogenetes
Cyanobacteria
Deferribacteres
Dictyoglomi
Thermodesulfobacteria
Thermotogae

Bacteria are a large domain of single-celled, prokaryote microorganisms. Typically a few micrometres in length, bacteria have a wide range of shapes, ranging from spheres to rods and spirals. Bacteria are ubiquitous in every habitat on Earth, growing in soil, acidic hot springs, radioactive waste, water, and deep in the Earth's crust, as well as in organic matter and the live bodies of plants and animals. There are typically 40 million bacterial cells in a gram of soil and a million bacterial cells in a millilitre of fresh water; in all, there are approximately five nonillion (5×10^{30}) bacteria on Earth, forming a biomass on Earth, which exceeds that of all plants and animals. Bacteria are vital in recycling nutrients, with many steps in nutrient cycles depending on these organisms, such as the fixation of nitrogen from the atmosphere and putrefaction. However, most bacteria have not been characterised, and only about half of the phyla of bacteria have species that can be grown in the laboratory. The study of bacteria is known as bacteriology, a branch of microbiology.

There are approximately ten times as many bacterial cells in the human flora as there are human cells in the body, with large numbers of bacteria on the skin and as gut flora. The vast majority of the bacteria in the body are rendered harmless by the protective effects of the immune system, and a few are beneficial. However, a few species of bacteria are pathogenic and cause infectious diseases, including cholera, syphilis, anthrax, leprosy and bubonic plague. The most common fatal bacterial diseases are respiratory infections, with tuberculosis alone killing about 2 million people a year, mostly in sub-Saharan Africa. In developed countries, antibiotics are used to treat bacterial infections and in agriculture, so antibiotic resistance is becoming common. In industry, bacteria are important in sewage

treatment, the production of cheese and yogurt through fermentation, as well as in biotechnology, and the manufacture of antibiotics and other chemicals.

Once regarded as plants constituting the Class Schizomycetes, bacteria are now classified as prokaryotes. Unlike cells of animals and other eukaryotes, bacterial cells do not contain a nucleus and rarely harbour membrane-bound organelles. Although the term *bacteria* traditionally included all prokaryotes, the scientific classification changed after the discovery in the 1990s that prokaryotes consist of two very different groups of organisms that evolved independently from an ancient common ancestor. These evolutionary domains are called Bacteria and Archaea.

Etymology

The word *bacteria* is the plural of the New Latin *bacterium*, which is the latinisation of the Greek βακτήριον (*baktērion*), the diminutive of βακτηρία (*baktēria*), meaning "staff, cane", because the first ones to be discovered were rod-shaped.

History of bacteriology

Antonie van Leeuwenhoek, the first microbiologist and the first person to observe bacteria using a microscope.

Bacteria were first observed by Antonie van Leeuwenhoek in 1676, using a single-lens microscope of his own design. He called them "animalcules" and published his observations in a series of letters to the Royal Society. The name *bacterium* was introduced much later, by Christian Gottfried Ehrenberg in 1838.

Louis Pasteur demonstrated in 1859 that the fermentation process is caused by the growth of microorganisms, and that this growth is not due to spontaneous generation. (Yeasts and molds, commonly associated with fermentation, are not bacteria, but rather fungi.) Along with his contemporary, Robert Koch, Pasteur was an early advocate of the germ theory of disease. Robert Koch was a pioneer in medical microbiology and worked on cholera, anthrax and tuberculosis. In his research into tuberculosis, Koch finally proved the germ theory, for which he was awarded a Nobel Prize in 1905. In *Koch's postulates*, he set out criteria to test if an organism is the cause of a disease, and these postulates are still used today.

Though it was known in the nineteenth century that bacteria are the cause of many diseases, no effective antibacterial treatments were available. In 1910, Paul Ehrlich developed the first antibiotic, by changing dyes that selectively stained *Treponema pallidum*—the spirochaete that causes syphilis—into compounds that selectively killed the pathogen. Ehrlich had been awarded a 1908 Nobel Prize for his work on immunology, and pioneered the use of stains to detect and identify bacteria, with his work being the basis of the Gram stain and the Ziehl-Neelsen stain.

A major step forward in the study of bacteria was the recognition in 1977 by Carl Woese that archaea have a separate line of evolutionary descent from bacteria. This new phylogenetic taxonomy was based on the sequencing of 16S ribosomal RNA, and divided prokaryotes into two evolutionary domains, as part of the three-domain system.

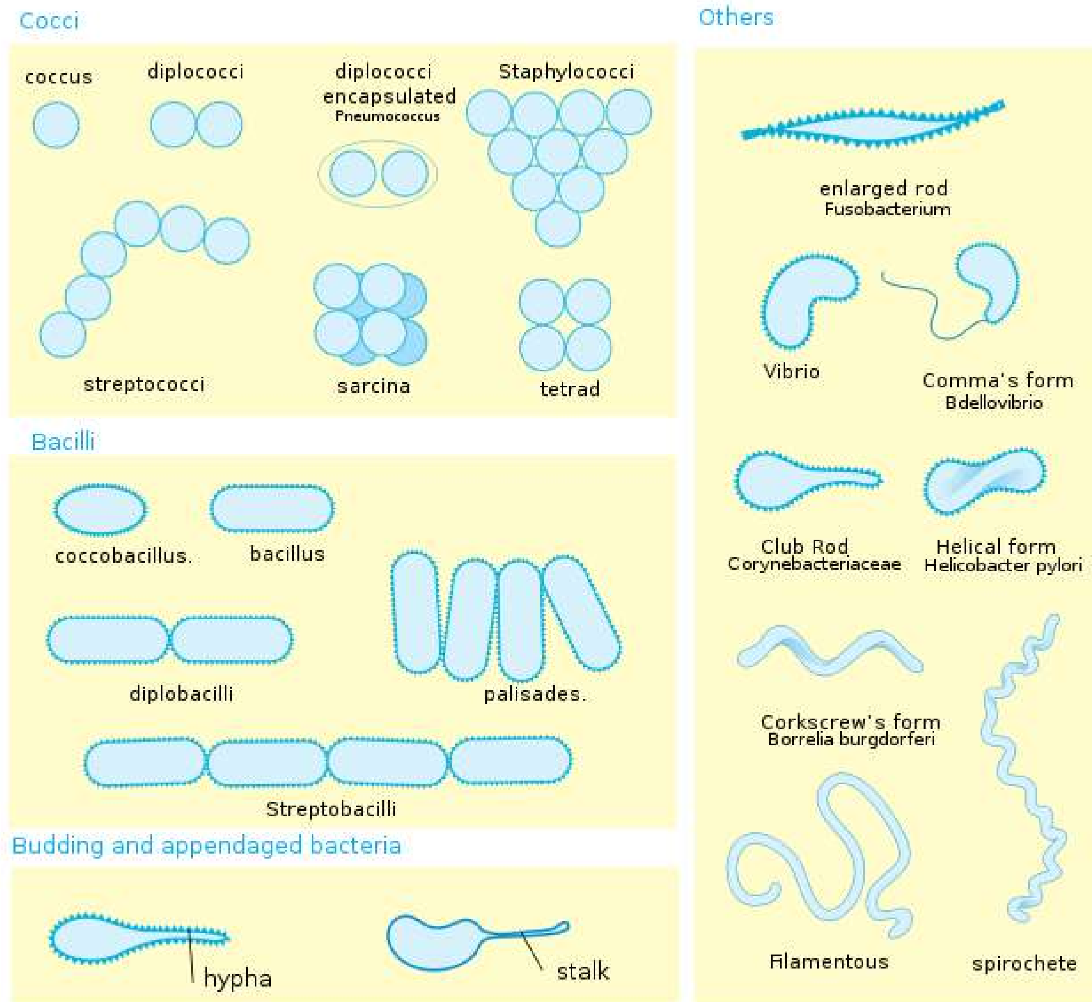
Origin and early evolution

The ancestors of modern bacteria were single-celled microorganisms that were the first forms of life to appear on Earth, about 4 billion years ago. For about 3 billion years, all organisms were microscopic, and bacteria and archaea were the dominant forms of life. Although bacterial fossils exist, such as stromatolites, their lack of distinctive morphology prevents them from being used to examine the history of bacterial evolution, or to date the time of origin of a particular bacterial species. However, gene sequences can be used to reconstruct the bacterial phylogeny, and these studies indicate that bacteria diverged first from the archaeal/eukaryotic lineage.

Bacteria were also involved in the second great evolutionary divergence, that of the archaea and eukaryotes. Here, eukaryotes resulted from ancient bacteria entering into endosymbiotic associations with the ancestors of eukaryotic cells, which were themselves possibly related to the Archaea. This involved the engulfment by proto-eukaryotic cells of alpha-proteobacterial symbionts to form either mitochondria or hydrogenosomes, which are still found in all known Eukarya (sometimes in highly reduced form, e.g. in ancient "amitochondrial" protozoa). Later on, some eukaryotes that already contained

mitochondria also engulfed cyanobacterial-like organisms. This led to the formation of chloroplasts in algae and plants. There are also some algae that originated from even later endosymbiotic events. Here, eukaryotes engulfed a eukaryotic algae that developed into a "second-generation" plastid. This is known as secondary endosymbiosis.

Morphology

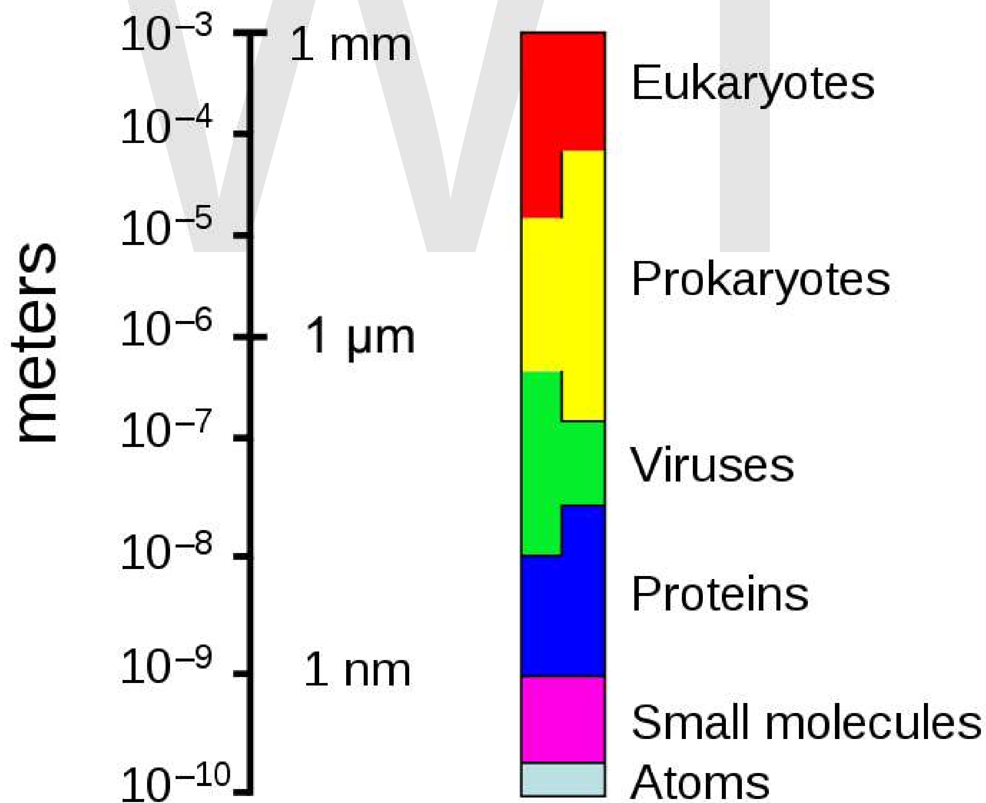


Bacteria display many cell morphologies and arrangements

Bacteria display a wide diversity of shapes and sizes, called *morphologies*. Bacterial cells are about one tenth the size of eukaryotic cells and are typically 0.5–5.0 micrometres in length. However, a few species–for example *Thiomargarita namibiensis* and *Epulopiscium fishelsoni*–are up to half a millimetre long and are visible to the unaided eye. Among the smallest bacteria are members of the genus *Mycoplasma*, which measure only 0.3 micrometres, as small as the largest viruses. Some bacteria may be even smaller, but these ultramicrobacteria are not well-studied.

Most bacterial species are either spherical, called cocci (*sing*. coccus, from Greek *κόκκος-kókkos*, grain, seed) or rod-shaped, called bacilli (*sing*. bacillus, from Latin *baculus*, stick). Elongation is associated with swimming. Some rod-shaped bacteria, called vibrio, are slightly curved or comma-shaped; others, can be spiral-shaped, called spirilla, or tightly coiled, called spirochaetes. A small number of species even have tetrahedral or cuboidal shapes. More recently, bacteria were discovered deep under the Earth's crust that grow as long rods with a star-shaped cross-section. The large surface area to volume ratio of this morphology may give these bacteria an advantage in nutrient-poor environments. This wide variety of shapes is determined by the bacterial cell wall and cytoskeleton, and is important because it can influence the ability of bacteria to acquire nutrients, attach to surfaces, swim through liquids and escape predators.

Many bacterial species exist simply as single cells, others associate in characteristic patterns: *Neisseria* form diploids (pairs), *Streptococcus* form chains, and *Staphylococcus* group together in "bunch of grapes" clusters. Bacteria can also be elongated to form filaments, for example the Actinobacteria. Filamentous bacteria are often surrounded by a sheath that contains many individual cells. Certain types, such as species of the genus *Nocardia*, even form complex, branched filaments, similar in appearance to fungal mycelia.

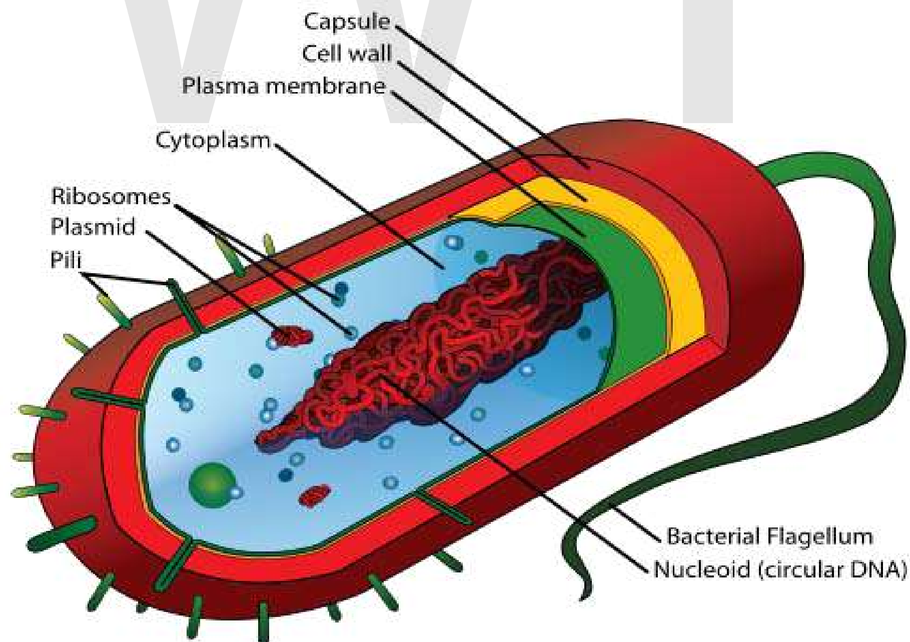


The range of sizes shown by prokaryotes, relative to those of other organisms and biomolecules

Bacteria often attach to surfaces and form dense aggregations called biofilms or bacterial mats. These films can range from a few micrometers in thickness to up to half a meter in depth, and may contain multiple species of bacteria, protists and archaea. Bacteria living in biofilms display a complex arrangement of cells and extracellular components, forming secondary structures such as microcolonies, through which there are networks of channels to enable better diffusion of nutrients. In natural environments, such as soil or the surfaces of plants, the majority of bacteria are bound to surfaces in biofilms. Biofilms are also important in medicine, as these structures are often present during chronic bacterial infections or in infections of implanted medical devices, and bacteria protected within biofilms are much harder to kill than individual isolated bacteria.

Even more complex morphological changes are sometimes possible. For example, when starved of amino acids, Myxobacteria detect surrounding cells in a process known as quorum sensing, migrate towards each other, and aggregate to form fruiting bodies up to 500 micrometres long and containing approximately 100,000 bacterial cells. In these fruiting bodies, the bacteria perform separate tasks; this type of cooperation is a simple type of multicellular organisation. For example, about one in 10 cells migrate to the top of these fruiting bodies and differentiate into a specialised dormant state called myxospores, which are more resistant to drying and other adverse environmental conditions than are ordinary cells.

Cellular structure



Structure and contents of a typical Gram positive bacterial cell

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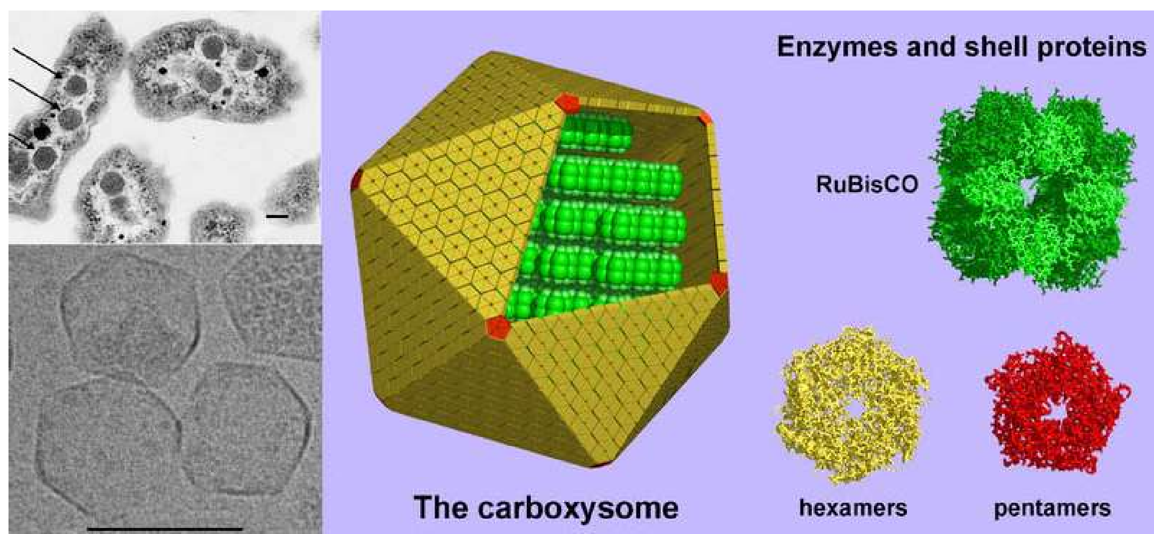
Intracellular structures

The bacterial cell is surrounded by a lipid membrane, or cell membrane, which encloses the contents of the cell and acts as a barrier to hold nutrients, proteins and other essential components of the cytoplasm within the cell. As they are prokaryotes, bacteria do not tend to have membrane-bound organelles in their cytoplasm and thus contain few large intracellular structures. They consequently lack a nucleus, mitochondria, chloroplasts and the other organelles present in eukaryotic cells, such as the Golgi apparatus and endoplasmic reticulum. Bacteria were once seen as simple bags of cytoplasm, but elements such as prokaryotic cytoskeleton, and the localization of proteins to specific locations within the cytoplasm have been found to show levels of complexity. These subcellular compartments have been called "bacterial hyperstructures".

Micro-compartments such as carboxysome provides a further level of organization, which are compartments within bacteria that are surrounded by polyhedral protein shells, rather than by lipid membranes. These "polyhedral organelles" localize and compartmentalize bacterial metabolism, a function performed by the membrane-bound organelles in eukaryotes.

Many important biochemical reactions, such as energy generation, occur by concentration gradients across membranes, a potential difference also found in a battery. The general lack of internal membranes in bacteria means reactions such as electron transport occur across the cell membrane between the cytoplasm and the periplasmic space. However, in many photosynthetic bacteria the plasma membrane is highly folded and fills most of the cell with layers of light-gathering membrane. These light-gathering complexes may even form lipid-enclosed structures called chlorosomes in green sulfur bacteria. Other proteins import nutrients across the cell membrane, or to expel undesired molecules from the cytoplasm.



Carboxysomes are protein-enclosed bacterial organelles. Top left is an electron microscope image of carboxysomes in *Halothiobacillus neapolitanus*, below is an image

of purified carboxysomes. On the right is a model of their structure. Scale bars are 100 nm.

Bacteria do not have a membrane-bound nucleus, and their genetic material is typically a single circular chromosome located in the cytoplasm in an irregularly shaped body called the nucleoid. The nucleoid contains the chromosome with associated proteins and RNA. The order Planctomycetes are an exception to the general absence of internal membranes in bacteria, because they have a membrane around their nucleoid and contain other membrane-bound cellular structures. Like all living organisms, bacteria contain ribosomes for the production of proteins, but the structure of the bacterial ribosome is different from those of eukaryotes and Archaea.

Some bacteria produce intracellular nutrient storage granules, such as glycogen, polyphosphate, sulfur or polyhydroxyalkanoates. These granules enable bacteria to store compounds for later use. Certain bacterial species, such as the photosynthetic Cyanobacteria, produce internal gas vesicles, which they use to regulate their buoyancy – allowing them to move up or down into water layers with different light intensities and nutrient levels.

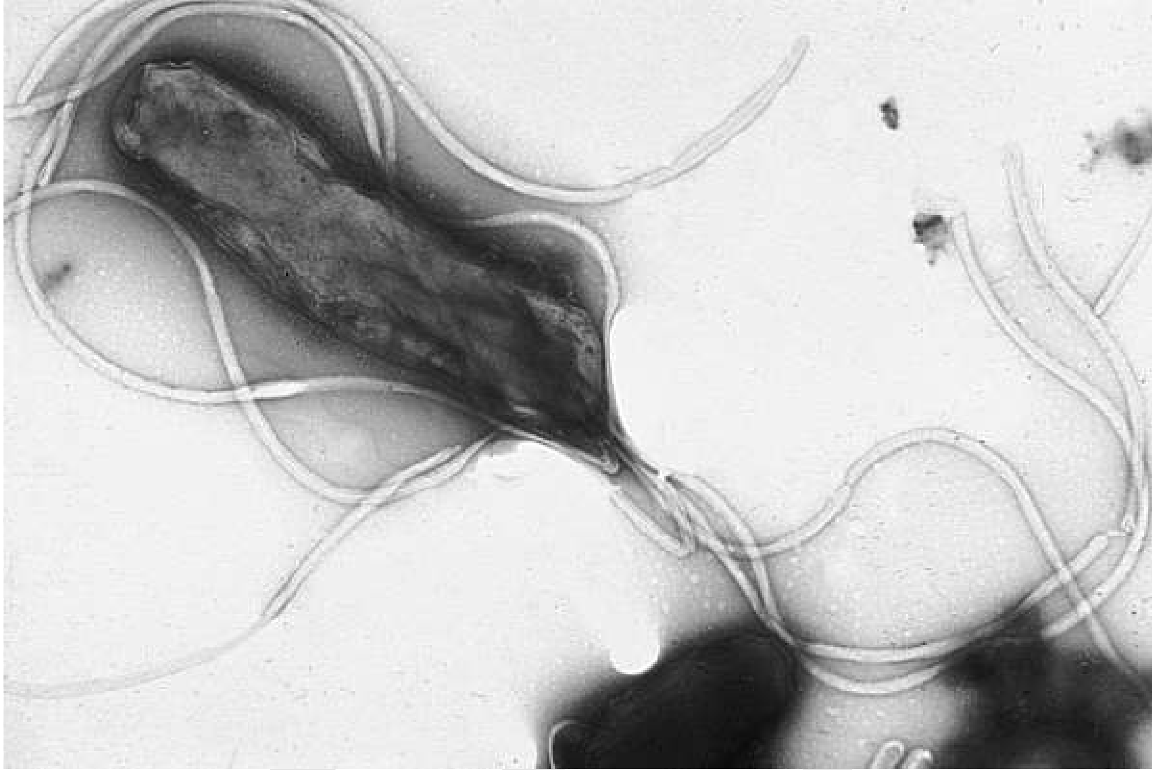
Extracellular structures

Around the outside of the cell membrane is the bacterial cell wall. Bacterial cell walls are made of peptidoglycan (called murein in older sources), which is made from polysaccharide chains cross-linked by unusual peptides containing D-amino acids. Bacterial cell walls are different from the cell walls of plants and fungi, which are made of cellulose and chitin, respectively. The cell wall of bacteria is also distinct from that of Archaea, which do not contain peptidoglycan. The cell wall is essential to the survival of many bacteria, and the antibiotic penicillin is able to kill bacteria by inhibiting a step in the synthesis of peptidoglycan.

There are broadly speaking two different types of cell wall in bacteria, called Gram-positive and Gram-negative. The names originate from the reaction of cells to the Gram stain, a test long-employed for the classification of bacterial species.

Gram-positive bacteria possess a thick cell wall containing many layers of peptidoglycan and teichoic acids. In contrast, Gram-negative bacteria have a relatively thin cell wall consisting of a few layers of peptidoglycan surrounded by a second lipid membrane containing lipopolysaccharides and lipoproteins. Most bacteria have the Gram-negative cell wall, and only the Firmicutes and Actinobacteria (previously known as the low G+C and high G+C Gram-positive bacteria, respectively) have the alternative Gram-positive arrangement. These differences in structure can produce differences in antibiotic susceptibility; for instance, vancomycin can kill only Gram-positive bacteria and is ineffective against Gram-negative pathogens, such as *Haemophilus influenzae* or *Pseudomonas aeruginosa*.

In many bacteria an S-layer of rigidly arrayed protein molecules covers the outside of the cell. This layer provides chemical and physical protection for the cell surface and can act as a macromolecular diffusion barrier. S-layers have diverse but mostly poorly understood functions, but are known to act as virulence factors in *Campylobacter* and contain surface enzymes in *Bacillus stearothermophilus*.



Helicobacter pylori electron micrograph, showing multiple flagella on the cell surface

Flagella are rigid protein structures, about 20 nanometres in diameter and up to 20 micrometres in length, that are used for motility. Flagella are driven by the energy released by the transfer of ions down an electrochemical gradient across the cell membrane.

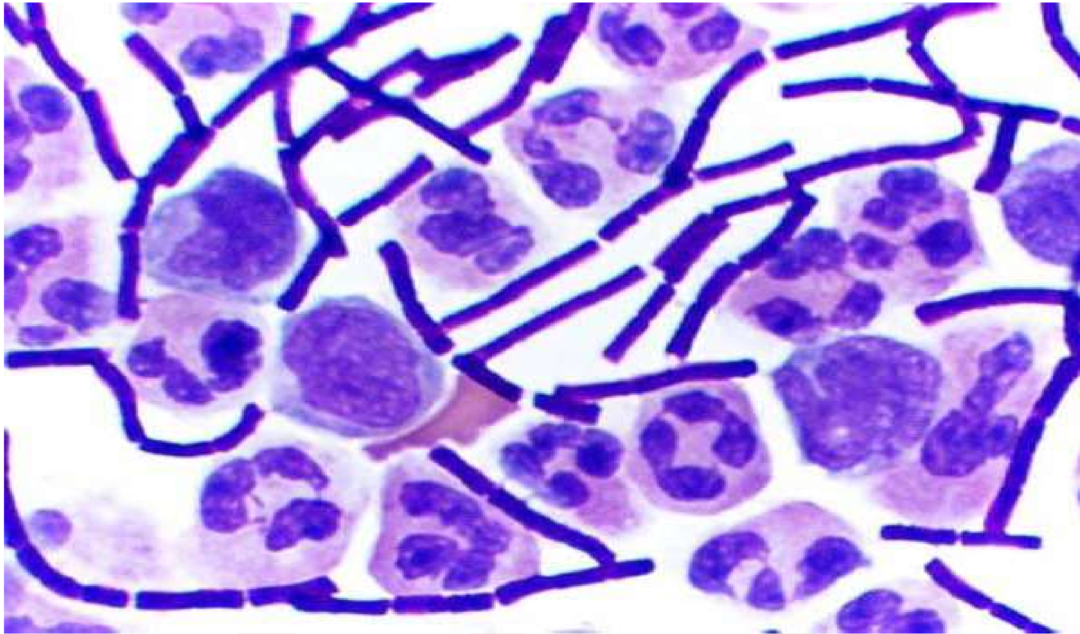
Fimbriae are fine filaments of protein, just 2–10 nanometres in diameter and up to several micrometers in length. They are distributed over the surface of the cell, and resemble fine hairs when seen under the electron microscope. Fimbriae are believed to be involved in attachment to solid surfaces or to other cells and are essential for the virulence of some bacterial pathogens. Pili (*sing*. pilus) are cellular appendages, slightly larger than fimbriae, that can transfer genetic material between bacterial cells in a process called conjugation.

Capsules or slime layers are produced by many bacteria to surround their cells, and vary in structural complexity: ranging from a disorganised slime layer of extra-cellular polymer, to a highly structured capsule or glycocalyx. These structures can protect cells from engulfment by eukaryotic cells, such as macrophages. They can also act as antigens

and be involved in cell recognition, as well as aiding attachment to surfaces and the formation of biofilms.

The assembly of these extracellular structures is dependent on bacterial secretion systems. These transfer proteins from the cytoplasm into the periplasm or into the environment around the cell. Many types of secretion systems are known and these structures are often essential for the virulence of pathogens, so are intensively studied.

Endospores



Bacillus anthracis (stained purple) growing in cerebrospinal fluid

Certain genera of Gram-positive bacteria, such as *Bacillus*, *Clostridium*, *Sporohalobacter*, *Anaerobacter* and *Heliobacterium*, can form highly resistant, dormant structures called endospores. In almost all cases, one endospore is formed and this is not a reproductive process, although *Anaerobacter* can make up to seven endospores in a single cell. Endospores have a central core of cytoplasm containing DNA and ribosomes surrounded by a cortex layer and protected by an impermeable and rigid coat.

Endospores show no detectable metabolism and can survive extreme physical and chemical stresses, such as high levels of UV light, gamma radiation, detergents, disinfectants, heat, freezing, pressure and desiccation. In this dormant state, these organisms may remain viable for millions of years, and endospores even allow bacteria to survive exposure to the vacuum and radiation in space. Endospore-forming bacteria can also cause disease: for example, anthrax can be contracted by the inhalation of *Bacillus anthracis* endospores, and contamination of deep puncture wounds with *Clostridium tetani* endospores causes tetanus.

Metabolism

Bacteria exhibit an extremely wide variety of metabolic types. The distribution of metabolic traits within a group of bacteria has traditionally been used to define their taxonomy, but these traits often do not correspond with modern genetic classifications. Bacterial metabolism is classified into nutritional groups on the basis of three major criteria: the kind of energy used for growth, the source of carbon, and the electron donors used for growth. An additional criterion of respiratory microorganisms are the electron acceptors used for aerobic or anaerobic respiration.

Nutritional types in bacterial metabolism

Nutritional type	Source of energy	Source of carbon	Examples
Phototrophs	Sunlight	Organic compounds (photoheterotrophs) or carbon fixation (photoautotrophs)	Cyanobacteria, Green sulfur bacteria, Chloroflexi, or Purple bacteria
Lithotrophs	Inorganic compounds	Organic compounds (lithoheterotrophs) or carbon fixation (lithoautotrophs)	Thermodesulfobacteria, *Hydrogenophilaceae*, or Nitrospirae
Organotrophs	Organic compounds	Organic compounds (chemoheterotrophs) or carbon fixation (chemoautotrophs)	*Bacillus*, *Clostridium* or *Enterobacteriaceae*

Carbon metabolism in bacteria is either heterotrophic, where organic carbon compounds are used as carbon sources, or autotrophic, meaning that cellular carbon is obtained by fixing carbon dioxide. Heterotrophic bacteria include parasitic types. Typical autotrophic bacteria are phototrophic cyanobacteria, green sulfur-bacteria and some purple bacteria, but also many chemolithotrophic species, such as nitrifying or sulfur-oxidising bacteria. Energy metabolism of bacteria is either based on phototrophy, the use of light through photosynthesis, or on chemotrophy, the use of chemical substances for energy, which are mostly oxidised at the expense of oxygen or alternative electron acceptors (aerobic/anaerobic respiration).

Filaments of photosynthetic cyanobacteria

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Finally, bacteria are further divided into lithotrophs that use inorganic electron donors and organotrophs that use organic compounds as electron donors. Chemotrophic organisms use the respective electron donors for energy conservation (by aerobic/anaerobic respiration or fermentation) and biosynthetic reactions (e.g. carbon dioxide fixation), whereas phototrophic organisms use them only for biosynthetic purposes. Respiratory organisms use chemical compounds as a source of energy by taking electrons from the reduced substrate and transferring them to a terminal electron acceptor in a redox reaction. This reaction releases energy that can be used to synthesise ATP and drive metabolism. In aerobic organisms, oxygen is used as the electron acceptor. In anaerobic organisms other inorganic compounds, such as nitrate, sulfate or carbon dioxide are used as electron acceptors. This leads to the ecologically important processes of denitrification, sulfate reduction and acetogenesis, respectively.

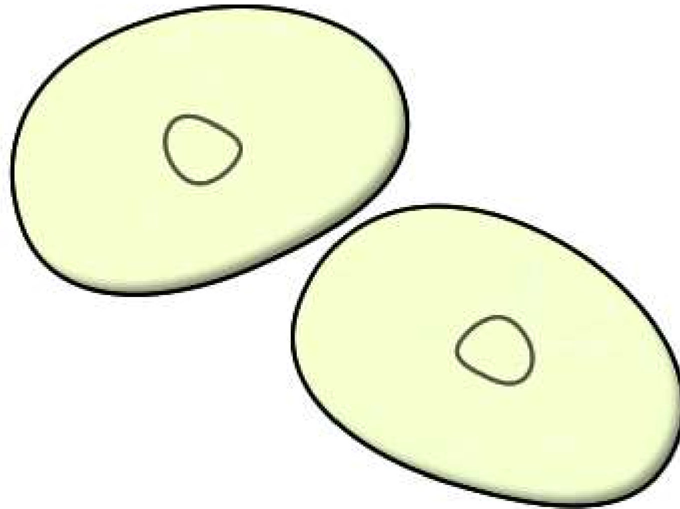
Another way of life of chemotrophs in the absence of possible electron acceptors is fermentation, where the electrons taken from the reduced substrates are transferred to oxidised intermediates to generate reduced fermentation products (e.g. lactate, ethanol, hydrogen, butyric acid). Fermentation is possible, because the energy content of the substrates is higher than that of the products, which allows the organisms to synthesise ATP and drive their metabolism.

These processes are also important in biological responses to pollution; for example, sulfate-reducing bacteria are largely responsible for the production of the highly toxic forms of mercury (methyl- and dimethylmercury) in the environment. Non-respiratory anaerobes use fermentation to generate energy and reducing power, secreting metabolic by-products (such as ethanol in brewing) as waste. Facultative anaerobes can switch between fermentation and different terminal electron acceptors depending on the environmental conditions in which they find themselves.

Lithotrophic bacteria can use inorganic compounds as a source of energy. Common inorganic electron donors are hydrogen, carbon monoxide, ammonia (leading to nitrification), ferrous iron and other reduced metal ions, and several reduced sulfur compounds. Unusually, the gas methane can be used by methanotrophic bacteria as both a source of electrons and a substrate for carbon anabolism. In both aerobic phototrophy and chemolithotrophy, oxygen is used as a terminal electron acceptor, while under anaerobic conditions inorganic compounds are used instead. Most lithotrophic organisms are autotrophic, whereas organotrophic organisms are heterotrophic.

In addition to fixing carbon dioxide in photosynthesis, some bacteria also fix nitrogen gas (nitrogen fixation) using the enzyme nitrogenase. This environmentally important trait can be found in bacteria of nearly all the metabolic types listed above, but is not universal.

Growth and reproduction



Many bacteria reproduce through *binary fission*

Unlike multicellular organisms, increases in the size of bacteria (cell growth) and their reproduction by cell division are tightly linked in unicellular organisms. Bacteria grow to a fixed size and then reproduce through binary fission, a form of asexual reproduction. Under optimal conditions, bacteria can grow and divide extremely rapidly, and bacterial populations can double as quickly as every 9.8 minutes. In cell division, two identical clone daughter cells are produced. Some bacteria, while still reproducing asexually, form more complex reproductive structures that help disperse the newly formed daughter cells. Examples include fruiting body formation by *Myxobacteria* and aerial hyphae formation by *Streptomyces*, or budding. Budding involves a cell forming a protrusion that breaks away and produces a daughter cell.

A colony of *Escherichia coli*.

In the laboratory, bacteria are usually grown using solid or liquid media. Solid growth media such as agar plates are used to isolate pure cultures of a bacterial strain. However, liquid growth media are used when measurement of growth or large volumes of cells are required. Growth in stirred liquid media occurs as an even cell suspension, making the cultures easy to divide and transfer, although isolating single bacteria from liquid media is difficult. The use of selective media (media with specific nutrients added or deficient, or with antibiotics added) can help identify specific organisms.

Most laboratory techniques for growing bacteria use high levels of nutrients to produce large amounts of cells cheaply and quickly. However, in natural environments nutrients are limited, meaning that bacteria cannot continue to reproduce indefinitely. This nutrient limitation has led the evolution of different growth strategies. Some organisms can grow extremely rapidly when nutrients become available, such as the formation of algal (and cyanobacterial) blooms that often occur in lakes during the summer. Other organisms have adaptations to harsh environments, such as the production of multiple antibiotics by *Streptomyces* that inhibit the growth of competing microorganisms. In nature, many organisms live in communities (e.g. biofilms) which may allow for increased supply of nutrients and protection from environmental stresses. These relationships can be essential for growth of a particular organism or group of organisms (syntrophy).

Bacterial growth follows three phases. When a population of bacteria first enter a high-nutrient environment that allows growth, the cells need to adapt to their new environment. The first phase of growth is the lag phase, a period of slow growth when the cells are adapting to the high-nutrient environment and preparing for fast growth. The lag phase has high biosynthesis rates, as proteins necessary for rapid growth are produced. The second phase of growth is the logarithmic phase (log phase), also known as the exponential phase. The log phase is marked by rapid exponential growth. The rate at which cells grow during this phase is known as the *growth rate* (k), and the time it takes the cells to double is known as the *generation time* (g). During log phase, nutrients are metabolised at maximum speed until one of the nutrients is depleted and starts limiting growth. The final phase of growth is the *stationary phase* and is caused by depleted nutrients. The cells reduce their metabolic activity and consume non-essential cellular proteins. The stationary phase is a transition from rapid growth to a stress response state and there is increased expression of genes involved in DNA repair, antioxidant metabolism and nutrient transport.

Genetics

Most bacteria have a single circular chromosome that can range in size from only 160,000 base pairs in the endosymbiotic bacteria *Candidatus Carsonella ruddii*, to 12,200,000 base pairs in the soil-dwelling bacteria *Sorangium cellulosum*. Spirochaetes of the genus *Borrelia* are a notable exception to this arrangement, with bacteria such as *Borrelia burgdorferi*, the cause of Lyme disease, containing a single linear chromosome. The genes in bacterial genomes are usually a single continuous stretch of DNA and although several different types of introns do exist in bacteria, these are much more rare than in eukaryotes.

Bacteria may also contain plasmids, which are small extra-chromosomal DNAs that may contain genes for antibiotic resistance or virulence factors.

Bacteria, as asexual organisms, inherit identical copies of their parent's genes (i.e., they are clonal). However, all bacteria can evolve by selection on changes to their genetic material DNA caused by genetic recombination or mutations. Mutations come from errors made during the replication of DNA or from exposure to mutagens. Mutation rates vary widely among different species of bacteria and even among different clones of a single species of bacteria. Genetic changes in bacterial genomes come from either random mutation during replication or "stress-directed mutation", where genes involved in a particular growth-limiting process have an increased mutation rate.

Some bacteria also transfer genetic material between cells. This can occur in three main ways. Firstly, bacteria can take up exogenous DNA from their environment, in a process called transformation. Genes can also be transferred by the process of transduction, when the integration of a bacteriophage introduces foreign DNA into the chromosome. The third method of gene transfer is bacterial conjugation, where DNA is transferred through direct cell contact. This gene acquisition from other bacteria or the environment is called horizontal gene transfer and may be common under natural conditions. Gene transfer is particularly important in antibiotic resistance as it allows the rapid transfer of resistance genes between different pathogens.

Bacteriophages

Bacteriophages are viruses that infect bacteria. Many types of bacteriophage exist, some simply infect and lyse their host bacteria, while others insert into the bacterial chromosome. A bacteriophage can contain genes that contribute to its host's phenotype: for example, in the evolution of *Escherichia coli* O157:H7 and *Clostridium botulinum*, the toxin genes in an integrated phage converted a harmless ancestral bacterium into a lethal pathogen. Bacteria resist phage infection through restriction modification systems that degrade foreign DNA, and a system that uses CRISPR sequences to retain fragments of the genomes of phage that the bacteria have come into contact with in the past, which allows them to block virus replication through a form of RNA interference. This CRISPR system provides bacteria with acquired immunity to infection.

Behavior

Secretion

Bacteria frequently secrete chemicals into their environment in order to modify it favorably. The secretions are often proteins and may act as enzymes that digest some form of food in the environment.

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Bioluminescence

A few bacteria have chemical systems that generate light. This bioluminescence often occurs in bacteria that live in association with fish, and the light probably serves to attract fish or other large animals.

Multicellularity

Bacteria often function as multicellular aggregates known as biofilms, exchanging a variety of molecular signals for inter-cell communication, and engaging in coordinated multicellular behavior.

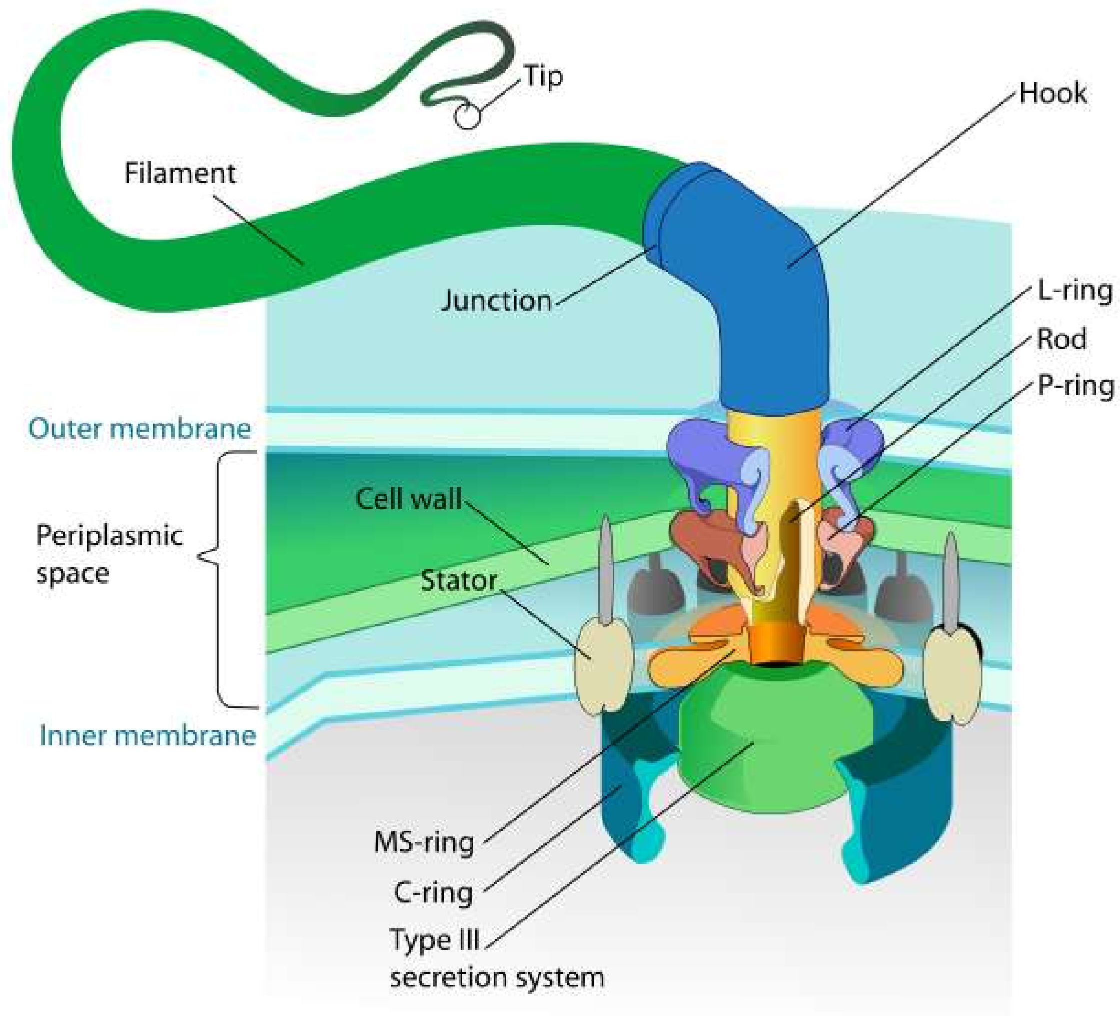
The communal benefits of multicellular cooperation include a cellular division of labor, accessing resources that cannot effectively be utilized by single cells, collectively defending against antagonists, and optimizing population survival by differentiating into distinct cell types. For example, bacteria in biofilms can have more than 500 times increased resistance to antibacterial agents than individual "planktonic" bacteria of the same species.

One type of inter-cellular communication by a molecular signal is called quorum sensing, which serves the purpose of determining whether there is a local population density that is sufficiently high that it is productive to invest in processes that are only successful if large numbers of similar organisms behave similarly, as in excreting digestive enzymes or emitting light.

Quorum sensing allows bacteria to coordinate gene expression, and enables them to produce, release and detect autoinducers or pheromones which accumulate with the growth in cell population.

Movement

Many bacteria can move using a variety of mechanisms: flagella are used for swimming through water; bacterial gliding and twitching motility move bacteria across surfaces; and changes of buoyancy allow vertical motion.



Flagellum of Gram-negative Bacteria. The base drives the rotation of the hook and filament.

Swimming bacteria frequently move near 10 body lengths per second and a few as fast as 100. This makes them at least as fast as fish, on a relative scale.

In twitching motility, bacterial use their type IV pili as a grappling hook, repeatedly extending it, anchoring it and then retracting it with remarkable force (>80 pN).

Flagella are semi-rigid cylindrical structures that are rotated and function much like the propeller on a ship. Objects as small as bacteria operate a low Reynolds number and cylindrical forms are more efficient than the flat, paddle-like, forms appropriate at human size scale.

Bacterial species differ in the number and arrangement of flagella on their surface; some have a single flagellum (monotrichous), a flagellum at each end (amphitrichous), clusters of flagella at the poles of the cell (lophotrichous), while others have flagella distributed over the entire surface of the cell (peritrichous). The bacterial flagella is the best-

understood motility structure in any organism and is made of about 20 proteins, with approximately another 30 proteins required for its regulation and assembly. The flagellum is a rotating structure driven by a reversible motor at the base that uses the electrochemical gradient across the membrane for power. This motor drives the motion of the filament, which acts as a propeller.

Many bacteria (such as *E. coli*) have two distinct modes of movement: forward movement (swimming) and tumbling. The tumbling allows them to reorient and makes their movement a three-dimensional random walk. The flagella of a unique group of bacteria, the spirochaetes, are found between two membranes in the periplasmic space. They have a distinctive helical body that twists about as it moves.

Motile bacteria are attracted or repelled by certain stimuli in behaviors called *taxes*: these include chemotaxis, phototaxis, energy taxis and magnetotaxis. In one peculiar group, the myxobacteria, individual bacteria move together to form waves of cells that then differentiate to form fruiting bodies containing spores. The myxobacteria move only when on solid surfaces, unlike *E. coli* which is motile in liquid or solid media.

Several *Listeria* and *Shigella* species move inside host cells by usurping the cytoskeleton, which is normally used to move organelles inside the cell. By promoting actin polymerization at one pole of their cells, they can form a kind of tail that pushes them through the host cell's cytoplasm.

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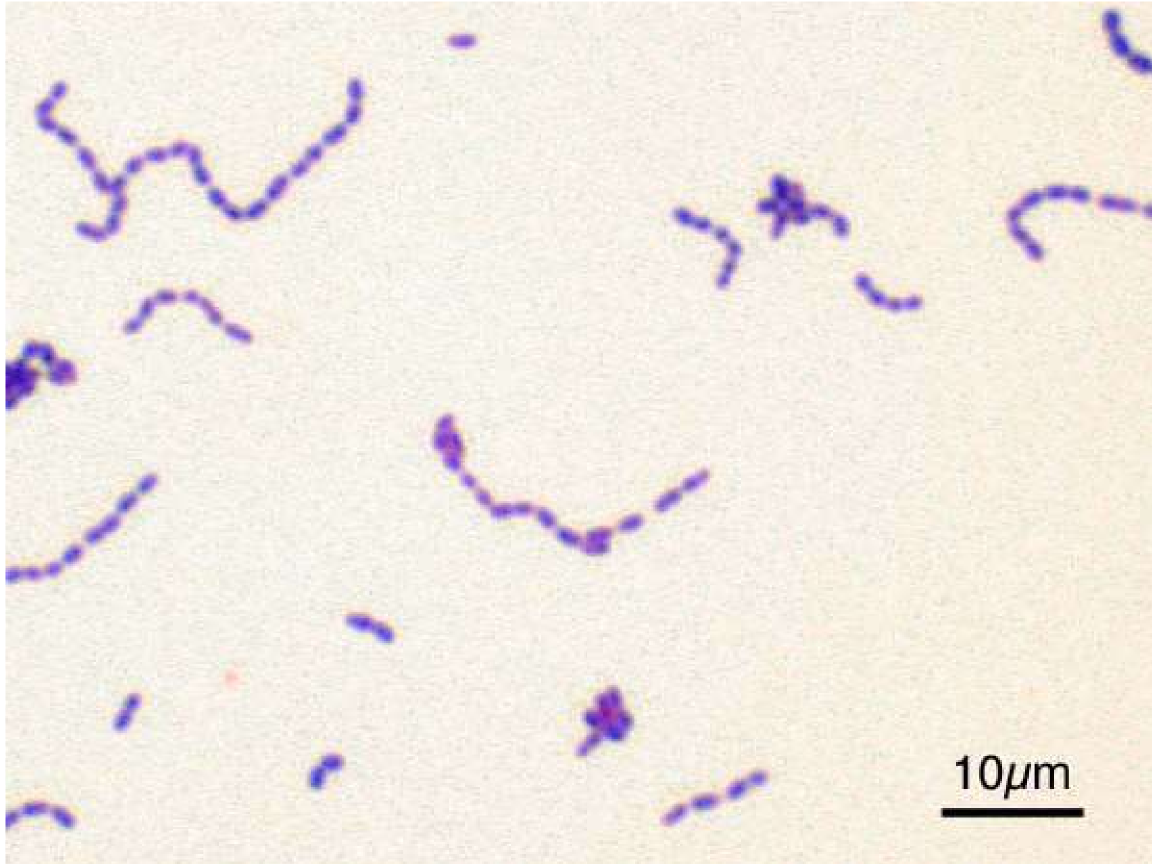
Classification and identification

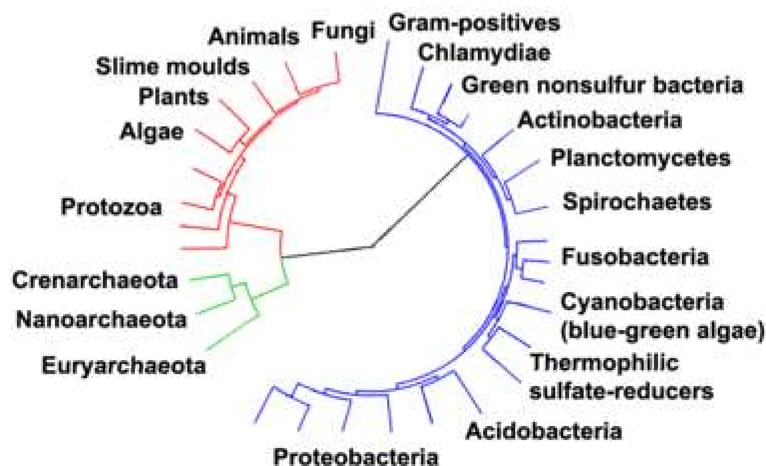


Streptococcus mutans visualized with a Gram stain

Classification seeks to describe the diversity of bacterial species by naming and grouping organisms based on similarities. Bacteria can be classified on the basis of cell structure, cellular metabolism or on differences in cell components such as DNA, fatty acids, pigments, antigens and quinones. While these schemes allowed the identification and classification of bacterial strains, it was unclear whether these differences represented variation between distinct species or between strains of the same species. This uncertainty was due to the lack of distinctive structures in most bacteria, as well as lateral gene transfer between unrelated species. Due to lateral gene transfer, some closely related bacteria can have very different morphologies and metabolisms. To overcome this uncertainty, modern bacterial classification emphasizes molecular systematics, using genetic techniques such as guanine cytosine ratio determination, genome-genome hybridization, as well as sequencing genes that have not undergone extensive lateral gene transfer, such as the rRNA gene. Classification of bacteria is determined by publication in the International Journal of Systematic Bacteriology, and Bergey's Manual of Systematic Bacteriology. The International Committee on Systematic Bacteriology (ICSB) maintains international rules for the naming of bacteria and taxonomic categories and for the ranking of them in the International Code of Nomenclature of Bacteria.

The term "bacteria" was traditionally applied to all microscopic, single-celled prokaryotes. However, molecular systematics showed prokaryotic life to consist of two separate domains, originally called *Eubacteria* and *Archaebacteria*, but now called *Bacteria* and *Archaea* that evolved independently from an ancient common ancestor. The archaea and eukaryotes are more closely related to each other than either is to the bacteria. These two domains, along with Eukarya, are the basis of the three-domain system, which is currently the most widely used classification system in microbiolology. However, due to the relatively recent introduction of molecular systematics and a rapid increase in the number of genome sequences that are available, bacterial classification remains a changing and expanding field. For example, a few biologists argue that the Archaea and Eukaryotes evolved from Gram-positive bacteria.

Identification of bacteria in the laboratory is particularly relevant in medicine, where the correct treatment is determined by the bacterial species causing an infection. Consequently, the need to identify human pathogens was a major impetus for the development of techniques to identify bacteria.



Phylogenetic tree showing the diversity of bacteria, compared to other organisms. Eukaryotes are colored red, archaea green and bacteria blue.

The Gram stain, developed in 1884 by Hans Christian Gram, characterises bacteria based on the structural characteristics of their cell walls. The thick layers of peptidoglycan in the "Gram-positive" cell wall stain purple, while the thin "Gram-negative" cell wall appears pink. By combining morphology and Gram-staining, most bacteria can be classified as belonging to one of four groups (Gram-positive cocci, Gram-positive bacilli, Gram-negative cocci and Gram-negative bacilli). Some organisms are best identified by stains other than the Gram stain, particularly mycobacteria or *Nocardia*, which show acid-fastness on Ziehl–Neelsen or similar stains. Other organisms may need to be identified by their growth in special media, or by other techniques, such as serology.

Culture techniques are designed to promote the growth and identify particular bacteria, while restricting the growth of the other bacteria in the sample. Often these techniques are designed for specific specimens; for example, a sputum sample will be treated to identify organisms that cause pneumonia, while stool specimens are cultured on selective media to identify organisms that cause diarrhoea, while preventing growth of non-pathogenic bacteria. Specimens that are normally sterile, such as blood, urine or spinal fluid, are cultured under conditions designed to grow all possible organisms. Once a pathogenic organism has been isolated, it can be further characterised by its morphology, growth patterns such as (aerobic or anaerobic growth, patterns of hemolysis) and staining.

As with bacterial classification, identification of bacteria is increasingly using molecular methods. Diagnostics using such DNA-based tools, such as polymerase chain reaction, are increasingly popular due to their specificity and speed, compared to culture-based methods. These methods also allow the detection and identification of "viable but nonculturable" cells that are metabolically active but non-dividing. However, even using these improved methods, the total number of bacterial species is not known and cannot even be estimated with any certainty. Following present classification, there are fewer than 9,000 known species of bacteria (including cyanobacteria), but attempts to estimate the true level of bacterial diversity have ranged from 10^7 to 10^9 total species – and even these diverse estimates may be off by many orders of magnitude.

Interactions with other organisms

Despite their apparent simplicity, bacteria can form complex associations with other organisms. These symbiotic associations can be divided into parasitism, mutualism and commensalism. Due to their small size, commensal bacteria are ubiquitous and grow on animals and plants exactly as they will grow on any other surface. However, their growth can be increased by warmth and sweat, and large populations of these organisms in humans are the cause of body odor.

Predators

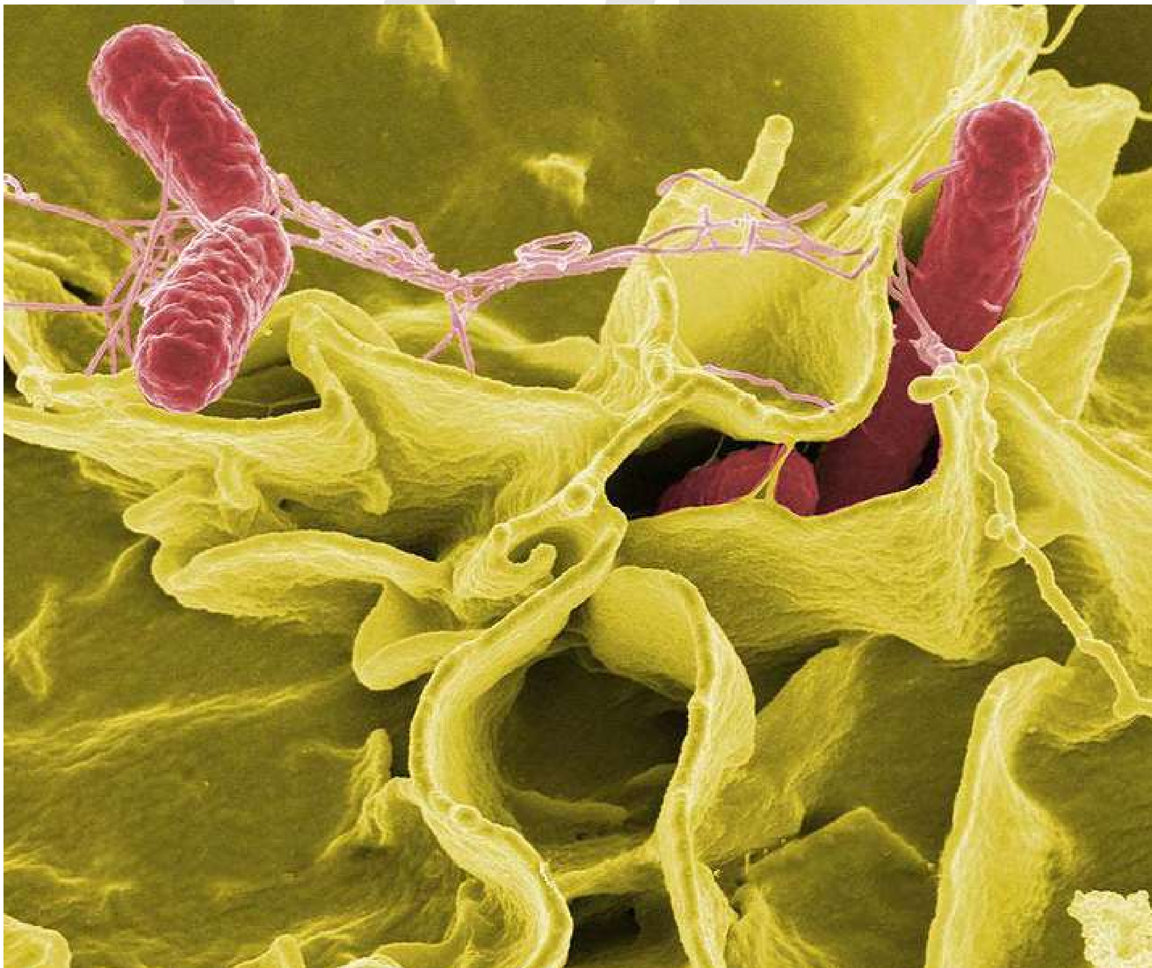
Some species of bacteria kill and then consume other microorganisms, these species called *predatory bacteria*. These include organisms such as *Myxococcus xanthus*, which forms swarms of cells that kill and digest any bacteria they encounter. Other bacterial predators either attach to their prey in order to digest them and absorb nutrients, such as *Vampirococcus*, or invade another cell and multiply inside the cytosol, such as *Daptobacter*. These predatory bacteria are thought to have evolved from saprophages that consumed dead microorganisms, through adaptations that allowed them to entrap and kill other organisms.

Mutualists

Certain bacteria form close spatial associations that are essential for their survival. One such mutualistic association, called interspecies hydrogen transfer, occurs between clusters of anaerobic bacteria that consume organic acids such as butyric acid or

propionic acid and produce hydrogen, and methanogenic Archaea that consume hydrogen. The bacteria in this association are unable to consume the organic acids as this reaction produces hydrogen that accumulates in their surroundings. Only the intimate association with the hydrogen-consuming Archaea keeps the hydrogen concentration low enough to allow the bacteria to grow.

In soil, microorganisms which reside in the rhizosphere (a zone that includes the root surface and the soil that adheres to the root after gentle shaking) carry out nitrogen fixation, converting nitrogen gas to nitrogenous compounds. This serves to provide an easily absorbable form of nitrogen for many plants, which cannot fix nitrogen themselves. Many other bacteria are found as symbionts in humans and other organisms. For example, the presence of over 1,000 bacterial species in the normal human gut flora of the intestines can contribute to gut immunity, synthesise vitamins such as folic acid, vitamin K and biotin, convert milk protein to lactic acid, as well as fermenting complex undigestible carbohydrates. The presence of this gut flora also inhibits the growth of potentially pathogenic bacteria (usually through competitive exclusion) and these beneficial bacteria are consequently sold as probiotic dietary supplements.



Color-enhanced scanning electron micrograph showing *Salmonella typhimurium* (red) invading cultured human cells

Pathogens

If bacteria form a parasitic association with other organisms, they are classed as pathogens. Pathogenic bacteria are a major cause of human death and disease and cause infections such as tetanus, typhoid fever, diphtheria, syphilis, cholera, foodborne illness, leprosy and tuberculosis. A pathogenic cause for a known medical disease may only be discovered many years after, as was the case with *Helicobacter pylori* and peptic ulcer disease. Bacterial diseases are also important in agriculture, with bacteria causing leaf spot, fire blight and wilts in plants, as well as Johne's disease, mastitis, salmonella and anthrax in farm animals.

Each species of pathogen has a characteristic spectrum of interactions with its human hosts. Some organisms, such as *Staphylococcus* or *Streptococcus*, can cause skin infections, pneumonia, meningitis and even overwhelming sepsis, a systemic inflammatory response producing shock, massive vasodilation and death. Yet these organisms are also part of the normal human flora and usually exist on the skin or in the nose without causing any disease at all. Other organisms invariably cause disease in humans, such as the Rickettsia, which are obligate intracellular parasites able to grow and reproduce only within the cells of other organisms. One species of Rickettsia causes typhus, while another causes Rocky Mountain spotted fever. *Chlamydia*, another phylum of obligate intracellular parasites, contains species that can cause pneumonia, or urinary tract infection and may be involved in coronary heart disease. Finally, some species such as *Pseudomonas aeruginosa*, *Burkholderia cenocepacia*, and *Mycobacterium avium* are opportunistic pathogens and cause disease mainly in people suffering from immunosuppression or cystic fibrosis.

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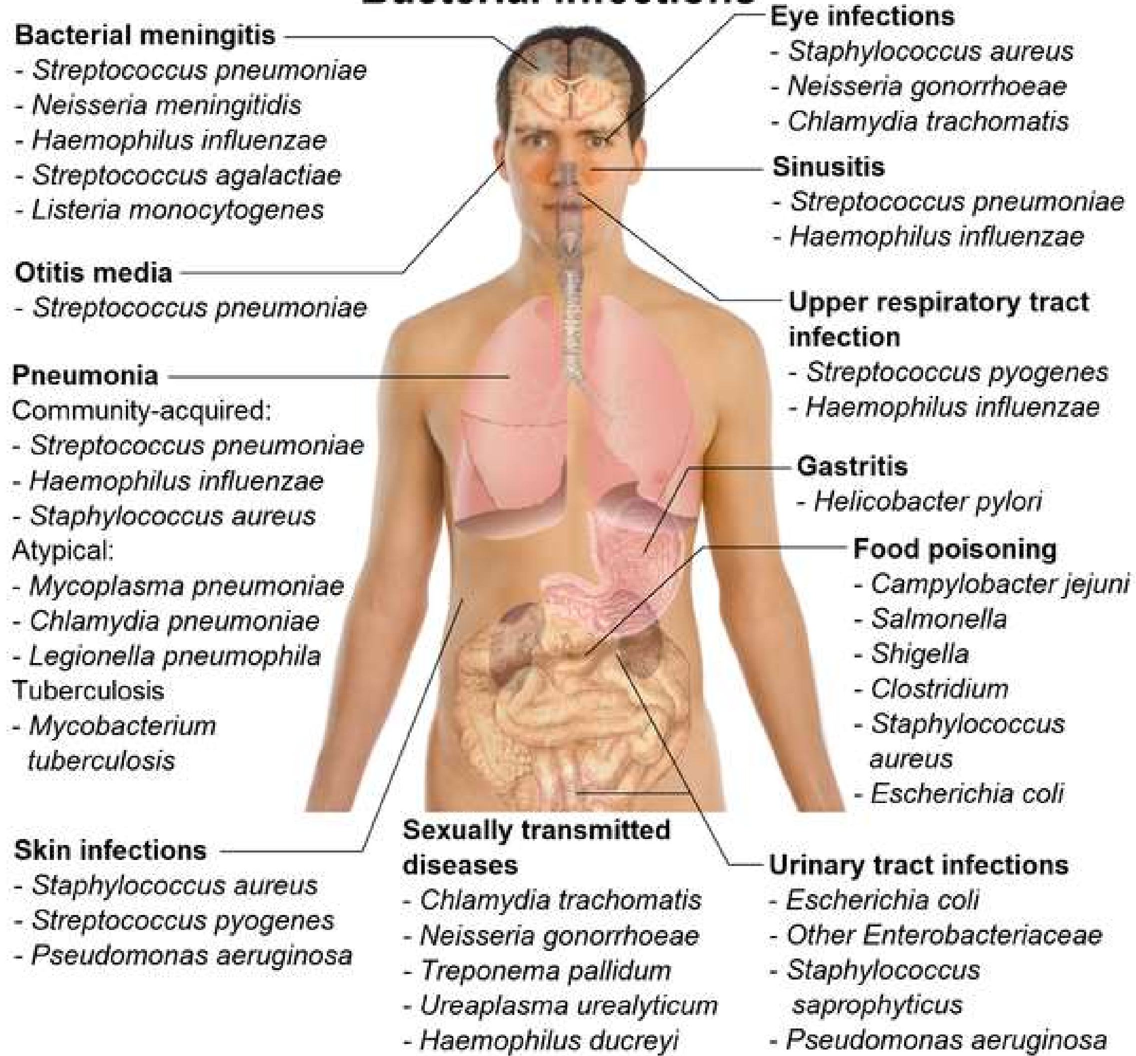

Overview of bacterial infections and main species involved.

Bacterial infections may be treated with antibiotics, which are classified as bacteriocidal if they kill bacteria, or bacteriostatic if they just prevent bacterial growth. There are many types of antibiotics and each class inhibits a process that is different in the pathogen from that found in the host. An example of how antibiotics produce selective toxicity are chloramphenicol and puromycin, which inhibit the bacterial ribosome, but not the structurally different eukaryotic ribosome. Antibiotics are used both in treating human disease and in intensive farming to promote animal growth, where they may be contributing to the rapid development of antibiotic resistance in bacterial populations. Infections can be prevented by antiseptic measures such as sterilizating the skin prior to piercing it with the needle of a syringe, and by proper care of indwelling catheters. Surgical and dental instruments are also sterilized to prevent contamination by bacteria. Disinfectants such as bleach are used to kill bacteria or other pathogens on surfaces to prevent contamination and further reduce the risk of infection.

Significance in technology and industry

Bacteria, often lactic acid bacteria such as *Lactobacillus* and *Lactococcus*, in combination with yeasts and molds, have been used for thousands of years in the preparation of fermented foods such as cheese, pickles, soy sauce, sauerkraut, vinegar, wine and yoghurt.

The ability of bacteria to degrade a variety of organic compounds is remarkable and has been used in waste processing and bioremediation. Bacteria capable of digesting the hydrocarbons in petroleum are often used to clean up oil spills. Fertilizer was added to some of the beaches in Prince William Sound in an attempt to promote the growth of these naturally occurring bacteria after the infamous 1989 *Exxon Valdez* oil spill. These efforts were effective on beaches that were not too thickly covered in oil. Bacteria are also used for the bioremediation of industrial toxic wastes. In the chemical industry, bacteria are most important in the production of enantiomerically pure chemicals for use as pharmaceuticals or agrichemicals.

Bacteria can also be used in the place of pesticides in the biological pest control. This commonly involves *Bacillus thuringiensis* (also called BT), a Gram-positive, soil dwelling bacterium. Subspecies of this bacteria are used as a Lepidopteran-specific insecticides under trade names such as Dipel and Thuricide. Because of their specificity, these pesticides are regarded as environmentally friendly, with little or no effect on humans, wildlife, pollinators and most other beneficial insects.

Because of their ability to quickly grow and the relative ease with which they can be manipulated, bacteria are the workhorses for the fields of molecular biology, genetics and biochemistry. By making mutations in bacterial DNA and examining the resulting phenotypes, scientists can determine the function of genes, enzymes and metabolic pathways in bacteria, then apply this knowledge to more complex organisms. This aim of understanding the biochemistry of a cell reaches its most complex expression in the synthesis of huge amounts of enzyme kinetic and gene expression data into mathematical models of entire organisms. This is achievable in some well-studied bacteria, with models of *Escherichia coli* metabolism now being produced and tested. This understanding of bacterial metabolism and genetics allows the use of biotechnology to bioengineer bacteria for the production of therapeutic proteins, such as insulin, growth factors, or antibodies.

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Chapter 6

Oral Microbiology

Oral microbiology is the study of the microorganisms of the oral cavity and the interactions between the oral microorganisms with each other and with the host. Of particular interest is the role of oral microorganisms in the two major dental diseases: dental caries and periodontal disease.

The mouth harbors a diverse, abundant and complex microbial community. This highly diverse microflora inhabits the various surfaces of the normal mouth. Bacteria accumulate on both the hard and soft oral tissues in biofilms. Bacterial adhesion is particularly important for oral bacteria.

Oral bacteria have evolved mechanisms to sense their environment and evade or modify the host. Bacteria occupy the ecological niche provided by both the tooth surface and gingival epithelium. However, a highly efficient innate host defense system constantly monitors the bacterial colonization and prevents bacterial invasion of local tissues. A dynamic equilibrium exists between dental plaque bacteria and the innate host defense system.

Oral bacteria

Oral bacteria include streptococci, lactobacilli, staphylococci, corynebacteria, and various anaerobes in particular bacteroides. The oral cavity of the new-born baby does not contain bacteria but rapidly becomes colonized with bacteria such as *Streptococcus salivarius*. With the appearance of the teeth during the first year colonization by *Streptococcus mutans* and *Streptococcus sanguis* occurs as these organisms colonise the dental surface and gingiva. Other strains of streptococci adhere strongly to the gums and cheeks but not to the teeth. The gingival crevice area (supporting structures of the teeth) provides a habitat for a variety of anaerobic species. Bacteroides and spirochetes colonize the mouth around puberty.

Treponema denticola

The levels of oral spirochetes are elevated in patients with periodontal diseases. Among this group, *Treponema denticola* is the most studied and is considered as one of the main etiological bacteria of periodontitis. *Treponema denticola* is a motile and highly proteolytic bacterium.

Fusospirochetes

Spirochetes and fusi-form bacilli live as normal flora in the mouth, but in the case of bleeding in the oral cavity, the bacteria can cause infection and diseases to oral cavity: 1/ Acute necrotizing ulcerative gingivitis (ANUG) 2/ Vincent angina with a membrane covering the throat area

Veillonella

Veillonella are gram-negative anaerobic cocci. It is thought that this species thrives in the acidic environment of caries and is thought to slow the development of dental caries. It converts the acidic products of other species to less acidic products.

Porphyromonas gingivalis

Porphyromonas gingivalis is a Gram-negative oral anaerobe strongly associated with chronic adult periodontitis. The bacterium produces a number of well-characterized virulence factors and can be manipulated genetically. The availability of the genome sequence is aiding our understanding of the biology of *P. gingivalis* and how it interacts with the environment, other bacteria and the human host.

Aggregatibacter actinomycetemcomitans

Aggregatibacter actinomycetemcomitans is considered an oral pathogen due to its virulence factors, its association with localized aggressive periodontitis in young adolescents, and studies indicating that it can cause bone loss.

Lactobacillus

Some *Lactobacillus* species have been associated with dental caries although these bacteria are normally symbiotic in humans and are found in the gut flora.

Dental plaque

Dental plaque is the material that adheres to the teeth and consists of bacterial cells (mainly *S. mutans* and *S. sanguis*), salivary polymers and bacterial extracellular products. Plaque is a biofilm on the surfaces of the teeth. This accumulation of microorganisms subject the teeth and gingival tissues to high concentrations of bacterial metabolites

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which results in dental disease. If not taken care of, via brushing or flossing, the plaque can turn into tartar (its hardened form)and lead to gingivitis or periodontal disease.

Cell-cell communication

Most of the bacterial species found in the mouth belong to microbial communities, called biofilms, a feature of which is inter-bacterial communication. Cell-cell contact, is mediated by specific protein adhesins and often, as in the case of inter-species aggregation, by complementary polysaccharide receptors. Another method of communication invoves cell-cell signalling molecules, which are of two classes: those used for intra-species and those used for inter-species signalling. An example of intra-species communication is quorum sensing. Oral bacteria have been shown to produce small peptides, such as competence stimulating peptides, which can help promote single-species biofilm formation. A common form of inter-species signalling is mediated by 4, 5-dihydroxy-2, 3-pentanedione (DPD) or Autoinducer-2 (Al-2).

Vaccination against oral infections

Dental caries and periodontitis have an infectious etiology and immunization has been proposed as a means of controlling them. However, the approaches vary according to the nature of the bacteria involved and the mechanisms of pathogenesis for these two very different diseases. In the case of dental caries, proteins involved in colonization of teeth by *Streptococcus mutans* can produce antibodies that inhibit the cariogenic process. Periodontal vaccines are less well developed, but some antigenic targets have been identified.

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Chapter 7

Pathogen

A **pathogen**, (from Greek: πάθος pathos "suffering, passion", and γἰγνομαι (γεν-) gignomai (gen-) "I give birth to") an **infectious agent**, or more commonly **germ**, is a biological agent such as a virus, bacteria, prion, or fungus that causes disease to its host. There are several substrates including *pathways* whereby pathogens can invade a host; the principal pathways have different episodic time frames, but soil contamination has the longest or most persistent potential for harboring a pathogen.

The body contains many natural orders of defense against some of the common pathogens (such as *Pneumocystis*) in the form of the human immune system and by some "helpful" bacteria present in the human body's normal flora. However, if the immune system or "good" bacteria is damaged in any way (such as by chemotherapy, human immunodeficiency virus (HIV), or antibiotics being taken to kill other pathogens), pathogenic bacteria that were being held at bay can proliferate and cause harm to the host. Such cases are called opportunistic infection.

Some pathogens (such as the bacterium *Yersinia pestis* which may have caused the Black Plague, the *Variola* virus, and the Malaria protozoa) have been responsible for massive numbers of casualties and have had numerous effects on afflicted groups. Of particular note in modern times is HIV, which is known to have infected several million humans globally, along with the Influenza virus. Today, while many medical advances have been made to safeguard against infection by pathogens, through the use of vaccination, antibiotics, and fungicide, pathogens continue to threaten human life. Social advances such as food safety, hygiene, and water treatment have reduced the threat from some pathogens. Not all pathogens are negative. In entomology, pathogens are one of the "Three P's" (predators, pathogens, and parasitoids) that serve as natural or introduced biological controls to suppress arthropod pest populations.

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Types of pathogen

Viral

Pathogenic viruses are mainly those of the families of: Adenoviridae, Picornaviridae, Herpesviridae, Hepadnaviridae, Flaviviridae, Retroviridae, Orthomyxoviridae, Paramyxoviridae, Papovaviridae, Polyomavirus, Rhabdoviridae, Togaviridae. Some notable pathogenic viruses cause: smallpox, influenza, mumps, measles, chickenpox, ebola, and rubella. Viruses typically range between 20-300 nanometers in length.

Bacterial

Although the vast majority of bacteria are harmless or beneficial, a few pathogenic bacteria can cause infectious diseases. The most common bacterial disease is tuberculosis, caused by the bacterium *Mycobacterium tuberculosis*, which affects about 2 million people mostly in sub-Saharan Africa. Pathogenic bacteria contribute to other globally important diseases, such as pneumonia, which can be caused by bacteria such as *Streptococcus* and *Pseudomonas*, and foodborne illnesses, which can be caused by bacteria such as *Shigella*, *Campylobacter* and *Salmonella*. Pathogenic bacteria also cause infections such as tetanus, typhoid fever, diphtheria, syphilis and Hansen's disease. Bacteria can often be killed by antibiotics because the cell wall in the outside is destroyed and then the D.N.A. They typically range between 1-5 micrometers in length.

Fungal

Fungi comprise a eukaryotic kingdom of microbes that are usually saprophytes but can cause diseases in humans, animals and plants. Fungi are the most common cause of diseases in crops and other plants. Life threatening fungal infections in humans most often occur in immunocompromised patients or vulnerable people with a weakened immune system, although fungi are common problems in the immunocompetent population as the causative agents of skin, nail or yeast infections. Most antibiotics that function on bacterial pathogens cannot be used to treat fungal infections because fungi and their hosts both have eukaryotic cells. Most clinical fungicides belong to the azole group. The typical fungal spore size is 1-40 micrometer in length.

Other parasites

Some eukaryotic organisms, such as protists and helminths, cause disease. One of the best known diseases caused by protists in the genus *Plasmodium* is malaria. These can range from 3-200 micrometers in length.

Prionic

Prions are infectious pathogens that do not contain nucleic acids. Prions are abnormal proteins whose presence causes some diseases such as scrapie, bovine spongiform

encephalopathy (mad cow disease) and Creutzfeldt–Jakob disease.. The discovery of prion as a new class of pathogen has lead Stanley B. Prusiner to receive Nobel Prize in Physiology or Medicine in 1997.

Potency

One hypothesis regarding pathogens states that the longer a pathogen can survive outside of the body, the more dangerous it can be to a potential host. For example, the smallpox virus (*variola virus*) can survive outside the human body for approximately 885 days. It is also one of the most deadly pathogenic viruses, as it kills between 20-50% of the people it infects. The tuberculosis bacterium kills 1 in 5 of the people it infects, but only survives 244 days outside of its host. However, research into the basis of the ability of pathogens to cause disease provides evidence from multiple and diverse species of the existence of pathogenicity or virulence factors, encoded within the pathogens' genetic material, that facilitate microbes to cause disease.

In countries that have higher sanitation standards, pathogens cannot survive for as long outside of the human. This is seen as encouragement to mutations to the pathogen which would make it less deadly, as such mutations would allow the pathogen to survive in the host for longer periods of time.

Transmission

One of the primary pathways by which food or water become contaminated is from the release of untreated sewage into a drinking water supply or onto cropland, with the result that people who eat or drink contaminated sources become infected. In developing countries most sewage is discharged into the environment or on cropland; even in developed countries there are periodic system failures resulting in a sanitary sewer overflow.

Examples of pathogens

Major human pathogens

- *Mycobacterium tuberculosis* — the causative agent of most cases of tuberculosis
- *Mycobacterium leprae* — the bacterium that causes leprosy (Hansen's disease)
- *Yersinia pestis* — pneumonic, septicemic, and the notorious bubonic plagues (aka "Black Death")
- *Rickettsia prowazekii* — the etiologic agent of typhus fever
- *Bartonella* spp.
- Spanish influenza virus

Chapter 8

Digital Holographic Microscopy

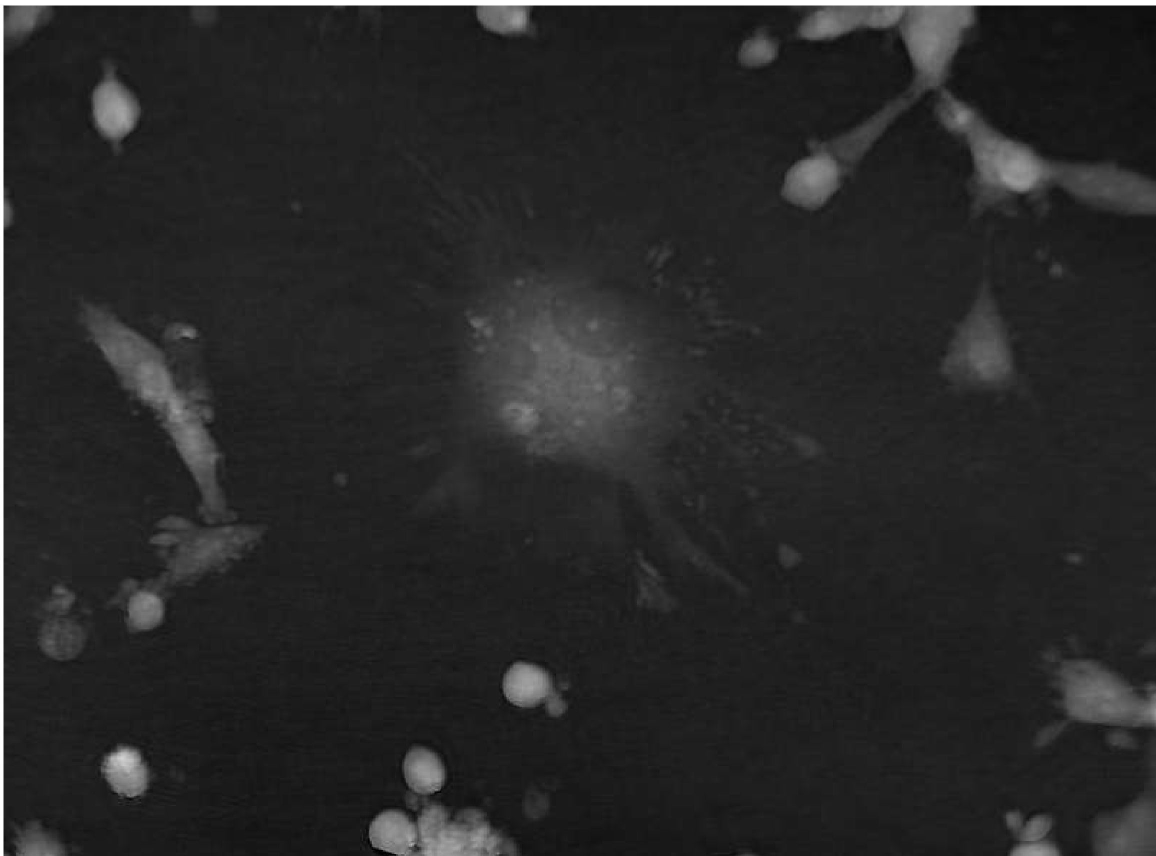

Figure 1. DHM phase shift image of cell details.

Digital holographic microscopy (DHM) is digital holography applied to microscopy. Digital holographic microscopy distinguishes itself from other microscopy methods by not recording the projected image of the object. Instead, the light wave front information originating from the object is digitally recorded as a hologram, from which a computer calculates the object image by using a numerical reconstruction algorithm. The image forming lens in traditional microscopy is thus replaced by a computer algorithm.

Other closely related microscopy methods to digital holographic microscopy are interferometric microscopy, optical coherence tomography and diffraction phase microscopy. Common to all methods is the use of a reference wave front to obtain amplitude (intensity) **and** phase information. The information is recorded on a digital image sensor or by a photo detector from which an image of the object is created (reconstructed) by a computer. In traditional microscopy, which do not use a reference wave front, only intensity information is recorded and essential information about the object is lost.

Digital holography has mostly been applied to light microscopy. However, digital holography has also been applied to electron microscopy. Holography was invented by Dennis Gabor to improve the electron microscope. For various reasons holography never made it into the electron microscope. Digital electron holography may finally bring home holography to its birth place and fulfill Gabor's vision.

Working principle

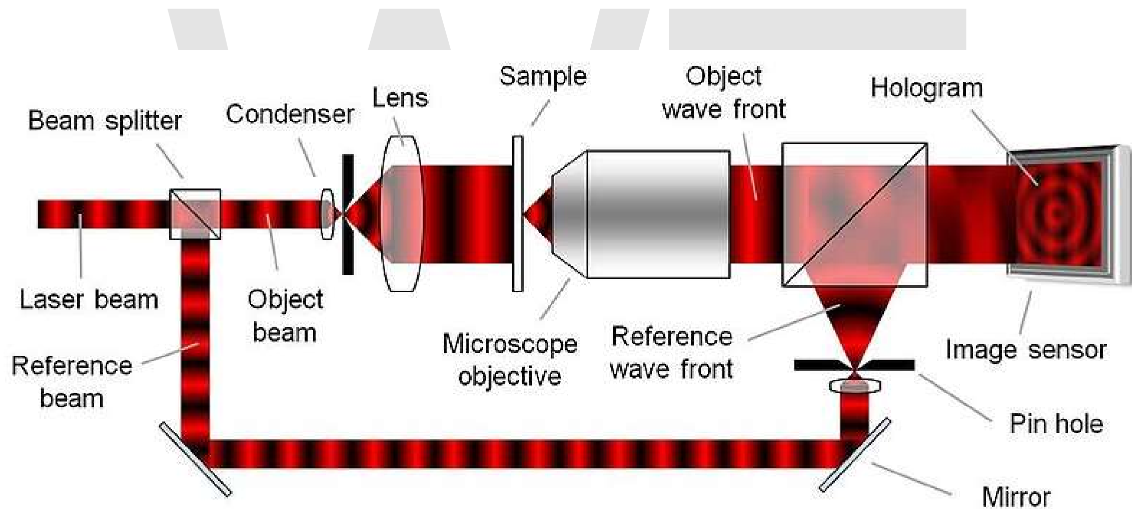

Figure 2. Typical optical setup of DHM.

To create the necessary interference pattern, i.e. the hologram, the illumination needs to be a coherent (monochromatic) light source, a laser for example. As can be seen in Figure 2, the laser light is split into an object beam and a reference beam. The expanded object beam illuminates the sample to create the object wave front. After the object wave front is collected by a microscope objective, the object and reference wave fronts are joined by a beam splitter to interfere and create the hologram. Using the digitally recorded hologram, a computer acts as a *digital lens* and calculates a viewable image of the object wave front by using a numerical reconstruction algorithm.

Commonly, a microscope objective is used to collect the object wave front. However, as the microscope objective is only used to collect light and not to form an image, it may be replaced by a simple lens. If a slightly lower optical resolution is acceptable, the microscope objective may be entirely removed.

Digital holography comes in different flavors, such as *off-axis Fresnel*, *Fourier*, *image plane*, *in-line*, *Gabor* and *phase-shifting* digital holography, depending on the optical setup. The basic principle, however, is the same; a hologram is recorded and an image is reconstructed by a computer.

The lateral optical resolution of digital holographic microscopy is equivalent to the resolution of traditional light microscopy. DHM is diffraction-limited by the numerical aperture, in the same way as traditional light microscopy. However, DHM offers a superb axial (depth) resolution. An axial accuracy of approximately 5 nm has been reported.

Advantages

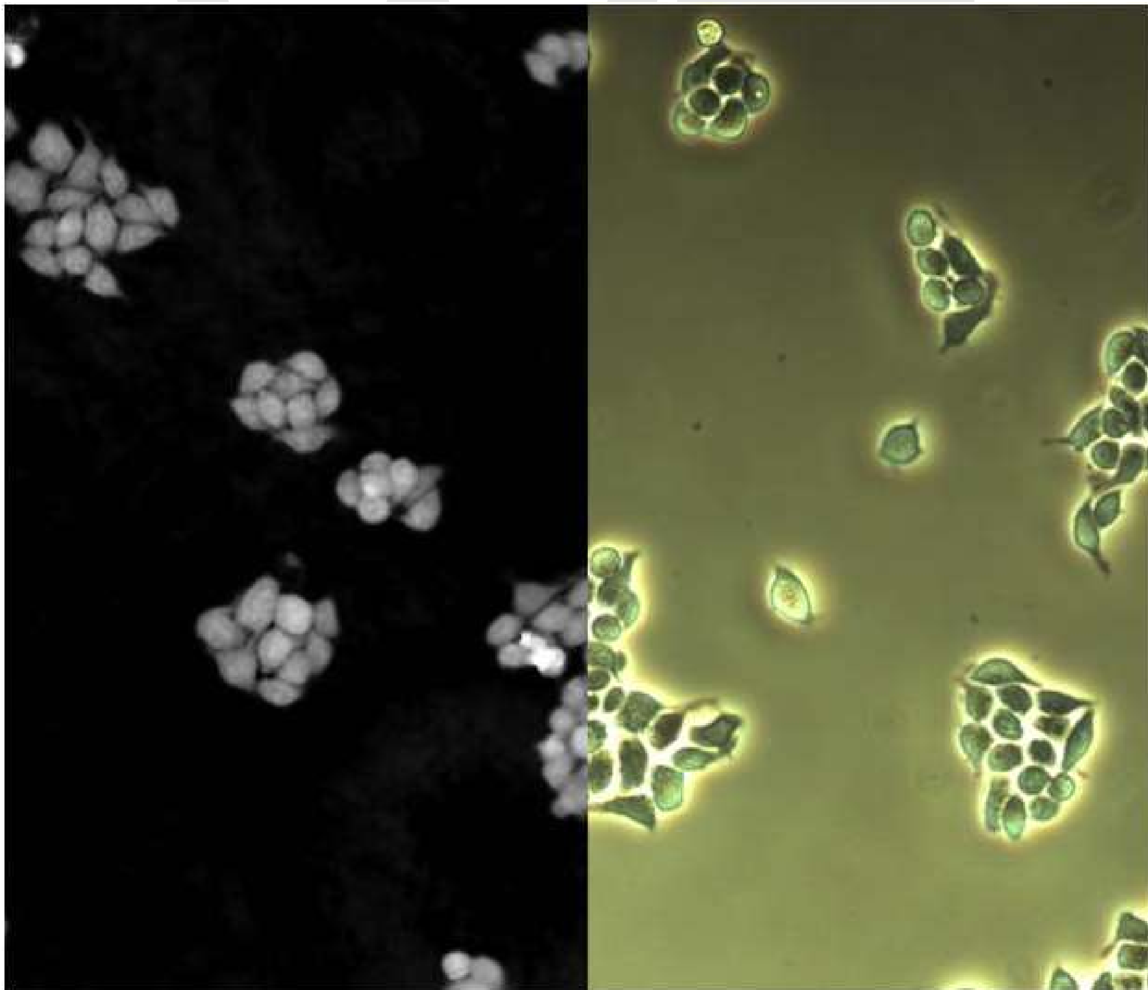

Figure 3. Comparison of a DHM phase shift image (left) and a phase contrast microscopy image (right).

Phase shift images

Besides the ordinary bright field image, a phase shift image is created as well. The phase shift image is unique for digital holographic microscopy and gives quantifiable information about optical distance. In reflection DHM, the phase shift image forms a topography image of the object.

Transparent objects, like living biological cells, are traditionally viewed in a phase contrast microscope or in a differential interference contrast microscope. These methods visualize phase shifting transparent objects by distorting the bright field image with phase shift information. Instead of distorting the bright field image, transmission DHM creates a separate phase shift image showing the optical thickness of the object. Digital holographic microscopy thus makes it possible to visualize and quantify transparent objects and is therefore also referred to as *quantitative phase contrast* microscopy.

Traditional phase contrast or bright field images of living unstained biological cells, Figure 3 (right), have proved themselves to be very difficult to analyze with image analysis software. On the contrary, phase shift images, Figure 3 (left), are readily segmented and analyzed by image analysis software based on mathematical morphology, such as CellProfiler.

3-Dimensional information

An object image is calculated at a given focal distance. However, as the recorded hologram contains all the necessary object wave front information, it is possible to calculate the object at any focal plane by changing the focal distance parameter in the reconstruction algorithm. In fact, the hologram contains all the information needed to calculate a complete image stack. In a DHM system, where the object wave front is recorded from multiple angles, it is possible to fully characterize the optical characteristics of the object and create tomography images of the object.

Digital autofocus

Conventional autofocus is achieved by vertically changing the focal distance until a focused image plane is found. As the complete stack of image planes may be calculated from a single hologram, it is possible to use any passive autofocus method to digitally select the focal plane. The digital auto focusing capabilities of digital holography opens up the possibility to scan and image surfaces extremely rapidly, without any vertical mechanical movement. By recording a single hologram and afterwards stitch sub-images together that are calculated at different focal planes, a complete and focused image of the object may be created.

Optical aberration correction

As DHM systems do not have an image forming lens, traditional optical aberrations do not apply to DHM. Optical aberrations are "corrected" by design of the reconstruction algorithm. A reconstruction algorithm that truly models the optical setup will not suffer from optical aberrations.

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Low cost

In optical microscopy systems, optical aberrations are traditionally corrected by combining lenses into a complex and costly image forming microscope objective. Furthermore, the narrow focal depth at high magnifications requires precision mechanics. The needed components for a DHM system are inexpensive optics and semiconductor components, such as a laser diode and an image sensor. The low component cost in combination with the auto focusing capabilities of DHM, make it possible to manufacture DHM systems for a very low cost.

Applications

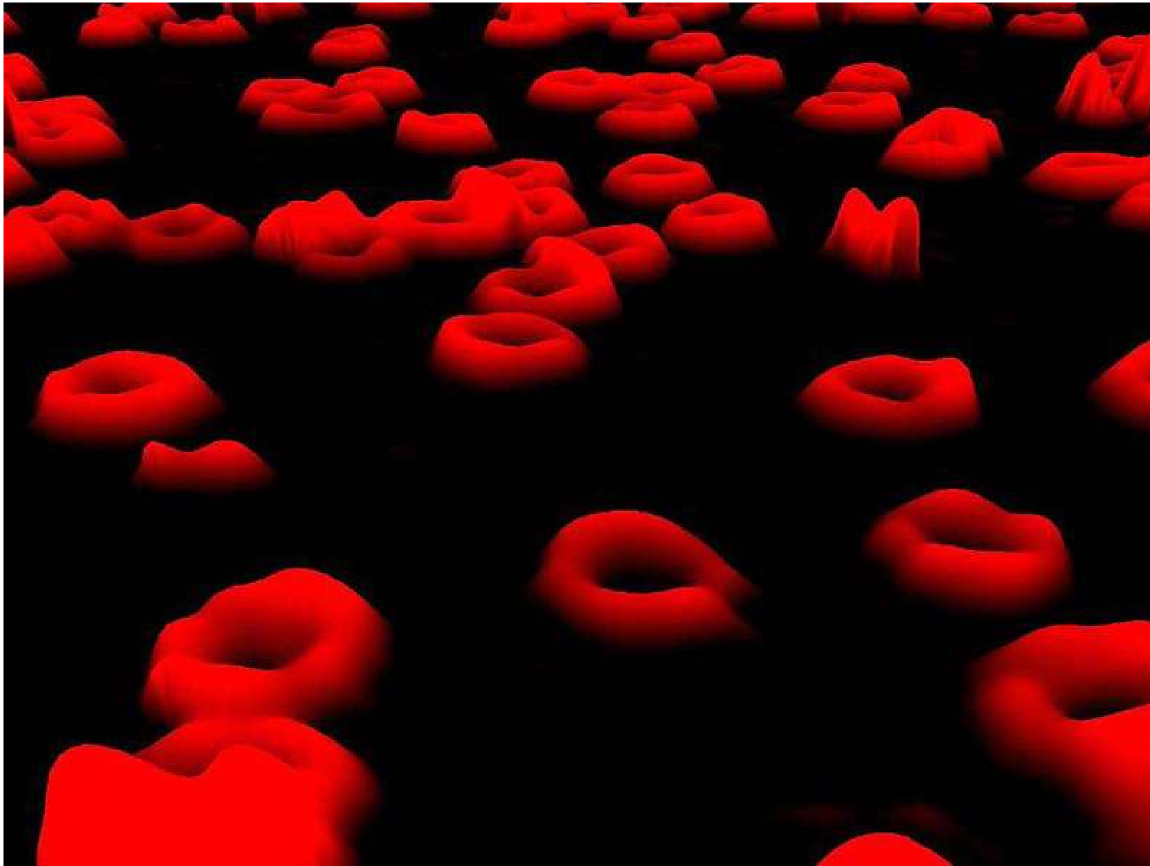

Figure 4. DHM phase shift image of human red blood cells.

Digital holographic microscopy has been successfully applied in a range of application areas. However, due to DHM's capability of non-invasively visualizing and quantifying biological tissue, bio-medical applications have received most attention. Examples of bio-medical applications are:

- **Label-free cell counting in adherent cell cultures.** Digital holographic microscopy makes it possible to perform cell counting and to measure cell

viability directly in the cell culture chamber. Today, the most commonly used cell counting methods, hemocytometer or Coulter counter, only work with cells that are in suspension.

- **Label-free viability analysis of adherent cell cultures.** Digital holography has been used to study the apoptotic process in different cell types. The refractive index changes taking place during the apoptotic process are easily measured with DHM.

- **Label-free cell cycle analysis.** The phase shift induced by cells has been shown to be correlated to the cell dry mass. The cell dry mass can be combined with other parameters obtainable by digital holography, such as cell volume and refractive index, to provide a better understanding of the cell cycle.

- **Label-free morphology analysis of cells.** Digital holography has been used in different contexts to study cell morphology using neither staining nor labeling. This can be used to follow processes such as the differentiation process where cell characteristics change. DHM has also been used for automated plant stem cell monitoring, and made it possible to distinguish between two types of stem cells by measuring morphological parameters.

- **Label free nerve cell studies.** Digital holographic microscopy makes it possible to study undisturbed processes in nerve cells as no labeling is required. The swelling and shape changing of nerve cells caused by cellular imbalance was easily studied.

- **Label-free high content analysis.** Fluorescent high content analysis/screening has several drawbacks. Label-free alternatives based on phase shift images have therefore been proposed. The capability of DHM to obtain phase shift images rapidly over large areas opens up new possibilities of very rapid quantitative characterization of the cell cycle and the effects of specific pharmacological agents.

- **Red blood cell analysis.** Phase shift images, created by diffraction phase microscopy, have been used to study red blood cell dynamics. Diffraction phase microscopy is very similar to digital holographic microscopy and creates phase shift images identical to the phase shift images created by digital holography.

- **Flow cytometry and particle tracking.** In-line digital holographic video microscopy has been used to analyze the radius and refractive index of particles in a microfluidic channel. These results can be applied on cells to enable real time digital holography flow cytometry.

- **Time-lapse microscopy of cell division and migration.** The autofocus and phase shift imaging capabilities of digital holographic microscopy makes it possible to

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effortlessly create label-free and quantifiable time-lapse. In Figure 5 a label-free time-lapse of dividing and migrating cells is shown.

- **Tomography studies.** Digital holographic microscopy allows for label-free and quantifiable analysis of subcellular motion deep in living tissue.

History

The first reports of replacing the photographic hologram of classical holography by digitally recording the hologram and numerically reconstructing the image in a computer was published in the late 1960s and in the early 1970s. Similar ideas were proposed for the electron microscope in the early 1980s. But, computers were too slow and recording capabilities were too poor for digital holography to be useful in practice. After the initial excitement, digital holography went into a similar hibernation as holography experienced about two decades earlier.

In the mid 1990s, digital image sensors and computers had become powerful enough to reconstruct images with some quality. In the 1960s, digital holography could either mean to compute an image from a hologram or to compute a hologram from a 3D model. The latter developed in parallel with classical holography during the hibernation of digital holography. During that time, digital holography was synonymous with what is now known as computer generated holography.

By the mid 1990s, digital image sensors and computers had improved tremendously, but still lacked the required performance for digital holography to be anything more than a curiosity. At the time, the market driving digital image sensors was primarily low-resolution video, and so those sensors provided only PAL, NTSC, or SECAM resolution. This suddenly changed at the beginning of the 21st century with the introduction of digital still image cameras, which drove demand for inexpensive high-pixel-count sensors. As of 2010, affordable image sensors can have up to 60 megapixels. In addition, the CD and DVD-player market has driven development of affordable diode lasers and optics.

The first reports of using digital holography for light microscopy came in the mid 1990s. However, it was not until the early 2000s that image sensor technology had progressed far enough to allow images of a reasonable quality. At that time, the first commercial digital holography companies also started to appear. With increased computing power and use of affordable high-resolution sensors and lasers, digital holographic microscopy became feasible and is finding applications, primarily within the life science.

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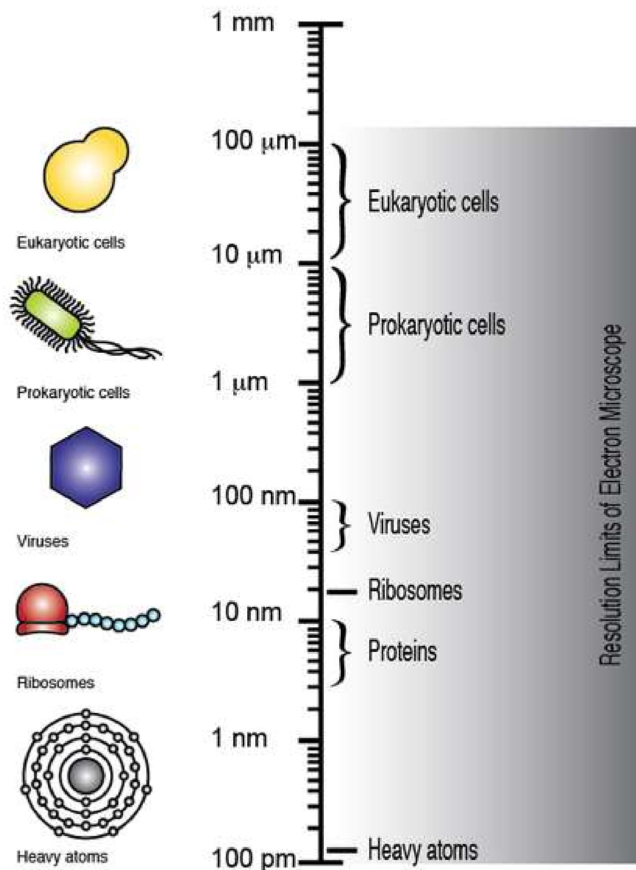
Chapter 9

Transmission Electron Microscopy DNA Sequencing



Resolution limits of the electron microscope The electron microscope can achieve a resolution of up to 100 picometers. Eukaryotic cells, prokaryotic cells, viruses, ribosomes, and even single atoms can be visualized. The scale is a logarithmic scale where each measurement represents a tenfold increase or decrease in length.

Transmission electron microscopy DNA sequencing is an emerging third-generation, single-molecule sequencing technology that uses transmission electron microscopy techniques. DNA is visible under the electron microscope; however, it must be labeled with heavy atoms so that the DNA bases can be clearly visualized. In addition, specialized imaging techniques and aberration corrected optics are beneficial for obtaining the resolution required to image the labeled DNA molecule. Transmission electron microscopy DNA sequencing advantageously may provide extremely long read lengths, but it is not yet commercially available.

History

Only a few years after James Watson and Francis Crick deduced the structure of DNA, and nearly two decades before Frederick Sanger published the first method for rapid DNA sequencing, Richard Feynman, an American physicist, envisioned the electron microscope as the tool that would one day allow biologists to "see the order of bases in the DNA chain". Feynman believed that if the electron microscope could be made powerful enough, then it would become possible to visualize the atomic structure of any and all chemical compounds, including DNA.

To this day, despite the invention of a multitude of chemical and fluorescent sequencing technologies, microscopy is still being explored as a means of performing single-molecule DNA sequencing. Two biotechnology companies have conceived of methods for high throughput, direct detection of DNA bases by transmission electron microscopy; however, these studies are still in their infancy and are far from being commercially available. The following progress in these technologies has been reported:

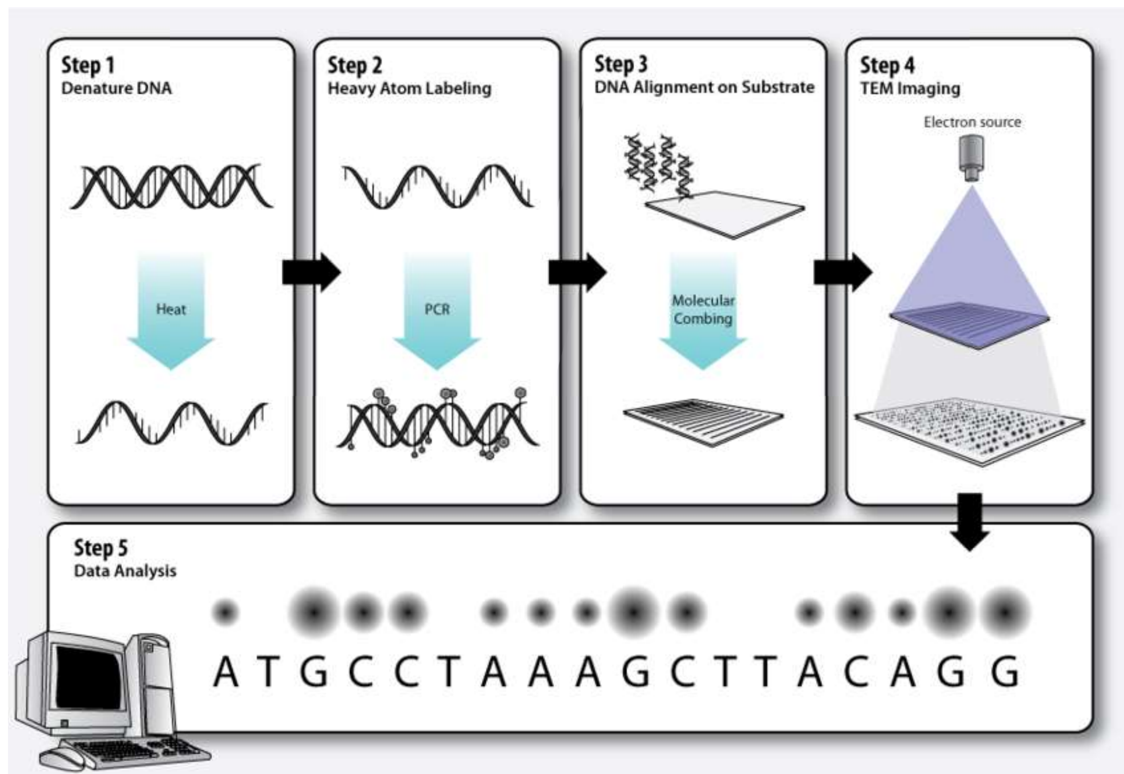
- **1970** Albert Crewe developed the high-angle annular dark-field imaging (HAADF) imaging technique in a scanning transmission electron microscope. Using this technique, he visualized individual heavy atoms on thin amorphous carbon films.
- **April 2008**: ZS Genetics presented its plans for development of a transmission electron microscopy-based single-molecule sequencing platform at the Cambridge Health-tech Institute (CHI) Sequencing Conference in San Diego, held from 23–24 April 2008.
- **March 2010**: Krivanek and colleagues reported several technical improvements to the HAADF method, including a combination of aberration corrected electron optics and low accelerating voltage. The latter is crucial for imaging biological objects, as it allows to reduce damage by the beam and increase the image contrast for light atoms. As a result, single atom substitutions in a boron nitride monolayer could be imaged. Halcyon Molecular is developing its single-molecule sequencing platform based on the technology utilized in this paper.
- **September 2010**: The Toste research group at University of California, Berkeley, received an Advanced Sequencing Technology Award from the National Human Genome Research Institute to continue research into single-molecule sequencing by transmission electron microscopy, in collaboration with Halcyon Molecular.

Principle

The electron microscope has the capacity to obtain a resolution of up to 100 pm, whereby microscopic biomolecules and structures such as viruses, ribosomes, proteins, lipids, small molecules and even single atoms can be observed.

Although DNA is visible when observed with the electron microscope, the resolution of the image obtained is not high enough to allow for deciphering the sequence of the individual bases, *i.e.*, DNA sequencing. However, upon differential labeling of the DNA bases with heavy atoms or metals, it is possible to both visualize and distinguish between the individual bases. Therefore, electron microscopy in conjunction with differential heavy atom DNA labeling could be used to directly image the DNA in order to determine its sequence.

Workflow



Workflow of transmission electron microscopy DNA sequencing

Step 1 – DNA denaturation

As in a standard polymerase chain reaction (PCR), the double stranded DNA molecules to be sequenced must be denatured before the second strand can be synthesized with labeled nucleotides.

Step 2 – Heavy atom labeling

The elements that make up biological molecules (C, H, N, O, P, S) are too light (low atomic number, Z) to be clearly visualized as individual atoms by transmission electron microscopy. To circumvent this problem, the DNA bases can be labeled with heavier atoms (higher Z). Each nucleotide is tagged with a characteristic heavy label, so that they can be distinguished in the transmission electron micrograph.

- ZS Genetics proposes using three heavy labels: bromine (Z=35), iodine (Z=53), and trichloromethane (total Z=63). These would appear as differential dark and light spots on the micrograph, and the fourth DNA base would remain unlabeled.
- Halcyon Molecular, in collaboration with the Toste group, proposes that purine and pyrimidine bases can be functionalized with platinum diamine or osmium tetraoxide bipyridine, respectively. Heavy metal atoms such as osmium (Z=76), iridium (Z=77), gold (Z=79), or uranium (Z=92) can then form metal-metal bonds with these functional groups to label the individual bases.

Step 3 – DNA alignment on substrate

The DNA molecules must be stretched out on a thin, solid substrate so that order of the labeled bases will be clearly visible on the electron micrograph. Molecular combing is a technique that utilizes the force of a receding air-water interface to extend DNA molecules, leaving them irreversibly bound to a silane layer once dry. This is one means by which alignment of the DNA on a solid substrate may be achieved.

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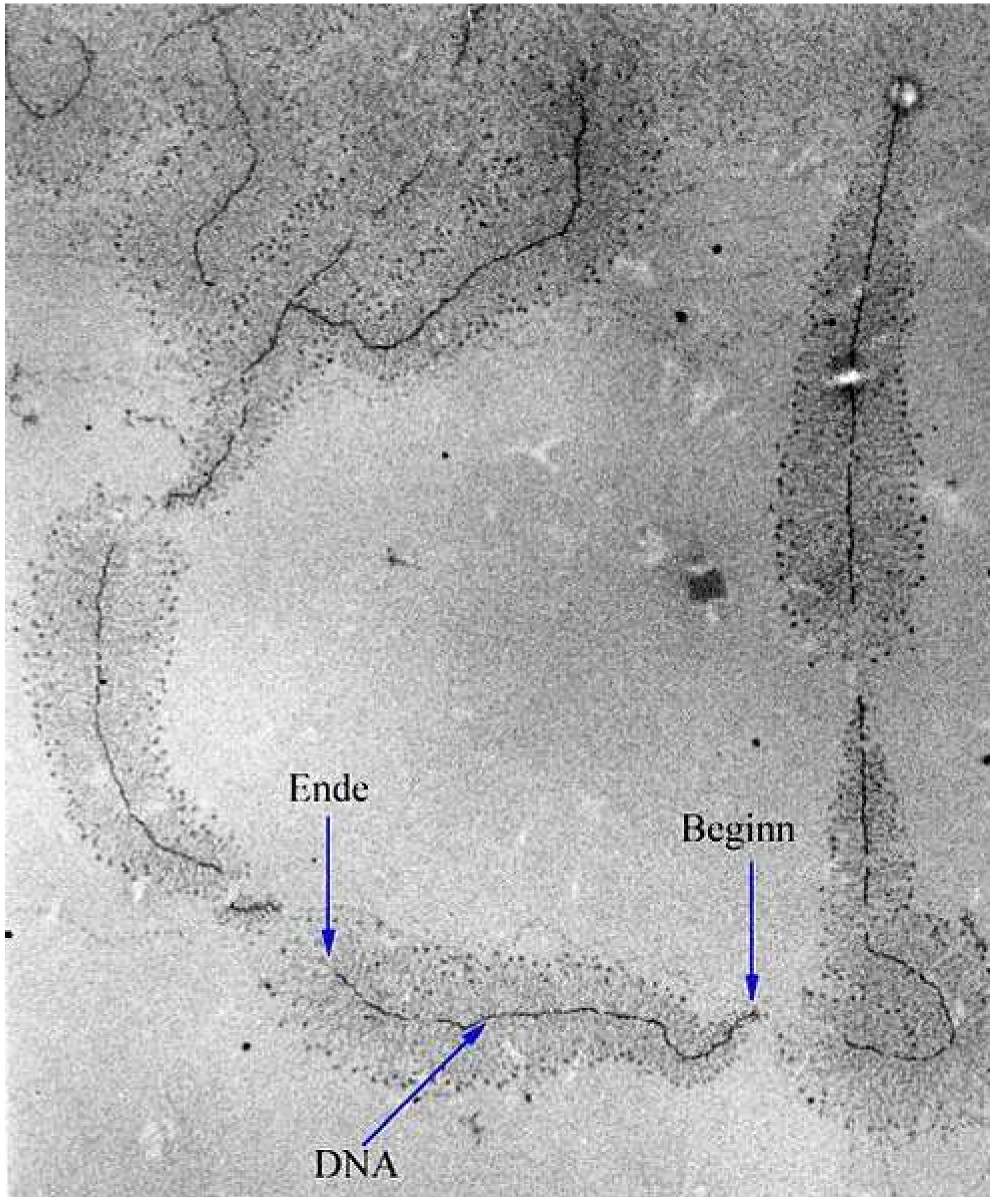
Step 4 – TEM imaging



Electron microscopy image of DNA: ribosomal transcription units of *Chrironumus pallidivitatus*. The image was recorded with the relatively old technology (ca. 2005).

Transmission electron microscopy (TEM) produces high magnification, high resolution images by passing a beam of electrons through a very thin sample. Whereas atomic resolution has been demonstrated with conventional TEM, further improvement in spatial resolution requires correcting the spherical and chromatic aberrations of the microscope lenses. This has only been possible in scanning transmission electron microscopy where the image is obtained by scanning the object with a finely focused electron beam, in a way similar to a cathode ray tube. However, the achieved improvement in resolution comes together with irradiation of the studied object by much higher beam intensities, the

concomitant sample damage and the associated imaging artefacts. Different imaging techniques are applied depending on whether the sample contains heavy or light atoms:

- Annular dark-field imaging measures the scattering of electrons as they deflect off the nuclei of the atoms in the transmission electron microscopy sample. This is best suited to samples containing heavy atoms, as they cause more scattering of electrons. The technique has been used to image atoms as light as boron, nitrogen, and carbon; however, the signal is very weak for such light atoms. If annular dark-field microscopy is put to use for transmission electron microscopy DNA sequencing, it will certainly be necessary to label the DNA bases with heavy atoms so that a strong signal can be detected.
- Annular bright-field imaging detects electrons transmitted directly through the sample, and measures the wave interference produced by their interactions with the atomic nuclei. This technique can detect light atoms with greater sensitivity than annular dark-field imaging methods. In fact, oxygen, nitrogen, lithium, and hydrogen in crystalline solids have been imaged using annular bright-field electron microscopy. Thus, it is theoretically possible to obtain direct images of the atoms in the DNA chain; however, the structure of DNA is much less geometric than crystalline solids, so direct imaging without prior labeling may not be achievable.

Step 5 – Data analysis

Dark and bright spots on the electron micrograph, corresponding to the differentially labeled DNA bases, are analyzed by computer software.

Applications

Transmission electron microscopy DNA sequencing is not yet commercially available, but the long read lengths that this technology may one day provide will make it useful in a variety of contexts.

De novo *genome assembly*

When sequencing a genome, it must be broken down into pieces that are short enough to be sequenced in a single read. These reads must then be put back together like a jigsaw puzzle by aligning the regions that overlap between reads; this process is called *de novo* genome assembly. The longer the read length that a sequencing platform provides, the longer the overlapping regions, and the easier it is to assemble the genome. From a computational perspective, microfluidic Sanger sequencing is still the most effective way to sequence and assemble genomes for which no reference genome sequence exists. The relatively long read lengths provide substantial overlap between individual sequencing reads, which allows for greater statistical confidence in the assembly. In addition, long Sanger reads are able to span most regions of repetitive DNA sequence which otherwise confound sequence assembly by causing false alignments. However, *de novo* genome assembly by Sanger sequencing is extremely expensive and time consuming. Second

generation sequencing technologies, while less expensive, are generally unfit for *de novo* genome assembly due to short read lengths. In general, third generation sequencing technologies, including transmission electron microscopy DNA sequencing, aim to improve read length while maintaining low sequencing cost. Thus, as third generation sequencing technologies improve, rapid and inexpensive *de novo* genome assembly will become a reality.

Full haplotypes

A haplotype is a series of linked alleles that are inherited together on a single chromosome. DNA sequencing can be used to genotype all of the single nucleotide polymorphisms (SNPs) that constitute a haplotype. However, short DNA sequencing reads often cannot be phased; that is, heterozygous variants cannot be confidently assigned to the correct haplotype. In fact, haplotyping with short read DNA sequencing data requires very high coverage (average >50x coverage of each DNA base) to accurately identify SNPs, as well as additional sequence data from the parents so that Mendelian transmission can be used to estimate the haplotypes. Sequencing technologies that generate long reads, including transmission electron microscopy DNA sequencing, can capture entire haploblocks in a single read. That is, haplotypes are not broken up among multiple reads, and the genetically linked alleles remain together in the sequencing data. Therefore, long reads make haplotyping easier and more accurate, which is beneficial to the field of population genetics.

Copy number variants

Genes are normally present in two copies in the diploid human genome; genes that deviate from this standard copy number are referred to as copy number variants (CNVs). Copy number variation can be benign (these are usually common variants, called copy number polymorphisms) or pathogenic. CNVs are detected by fluorescence in situ hybridization (FISH) or comparative genomic hybridization (CGH). To detect the specific breakpoints at which a deletion occurs, or to detect genomic lesions introduced by a duplication or amplification event, CGH can be performed using a tiling array (array CGH), or the variant region can be sequenced. Long sequencing reads are especially useful for analyzing duplications or amplifications, as it is possible to analyze the orientation of the amplified segments if they are captured in a single sequencing read.

Cancer

Cancer genomics, or oncogenomics, is an emerging field in which high-throughput, second generation DNA sequencing technology is being applied to sequence entire cancer genomes. Analyzing this short read sequencing data encompasses all of the problems associated with *de novo* genome assembly using short read data. Furthermore, cancer genomes are often aneuploid. These aberrations, which are essentially large scale copy number variants, can be analyzed by second-generation sequencing technologies using read frequency to estimate the copy number. Longer reads would, however, provide

a more accurate picture of copy number, orientation of amplified regions, and SNPs present in cancer genomes.

Microbiome sequencing

The microbiome refers the total collection of microbes present in a microenvironment and their respective genomes. For example, an estimated 100 trillion microbial cells colonize the human body at any given time. The human microbiome is of particular interest, as these commensal bacteria are important for human health and immunity. Most of the Earth's bacterial genomes have not yet been sequenced; undertaking a microbiome sequencing project would require extensive *de novo* genome assembly, a prospect which is daunting with short read DNA sequencing technologies. Longer reads would greatly facilitate the assembly of new microbial genomes.

Strengths and weaknesses

Compared to other second- and third-generation DNA sequencing technologies, transmission electron microscopy DNA sequencing has a number of potential key strengths and weaknesses, which will ultimately determine its usefulness and prominence as a future DNA sequencing technology.

Strengths

- **Longer read lengths**: ZS Genetics has estimated potential read lengths of transmission electron microscopy DNA sequencing to be 10,000 to 20,000 base pairs with a rate of 1.7 billion base pairs per day. Such long read lengths would allow easier *de novo* genome assembly and direct detection of haplotypes, among other applications.
- **Lower cost**: Transmission electron microscopy DNA sequencing is estimated to cost just US$5,000-US$10,000 per human genome, compared to the more expensive second-generation DNA sequencing alternatives.
- **No dephasing**: Dephasing of the DNA strands due to loss in synchronicity during synthesis is a major problem of second-generation sequencing technologies. For transmission electron microscopy DNA sequencing and several other third-generation sequencing technologies, sychronization of the reads is unnecessary as only one molecule is being read at a time.
- **Shorter turnaround time**: The capacity to read native fragments of DNA renders complex template preparation an unnecessary step in the general workflow of whole genome sequencing. Consequently, shorter turnaround times are possible.

Weaknesses

- **High capital cost**: A transmission electron microscope with sufficient resolution required for transmission electron microscopy DNA sequencing costs approximately US$1,000,000, therefore pursuing DNA sequencing by this method requires a substantial investment.

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- **Technically challenging**: Selective heavy atom labeling and attaching and straightening the labeled DNA to a substrate are a serious technical challenge. Further, the DNA sample should be stable to the high vacuum of electron microscope and irradiation by a focused beam of high-energy electrons.
- **Potential PCR bias and artefacts**: Although PCR is only being utilized in transmission electron microscopy DNA sequencing as a means to label the DNA strand with heavy atoms or metals, there could be the possibility of introducing bias in template representation or errors during the single amplification.

Comparison to other sequencing technologies

Many non-Sanger second- and third-generation DNA sequencing technologies have been or are currently being developed with the common aim of increasing throughput and decreasing cost such that personalized genetic medicine can be fully realized.

Both the US$10 million Archon X Prize for Genomics supported by the X Prize Foundation (Santa Monica, CA, USA) and the US$70 million in grant awards supported by the National Human Genome Research Institute of the National Institutes of Health (NIH-NHGRI) are fueling the rapid burst of research activity in the development of new DNA sequencing technologies.

Since different approaches, techniques, and strategies are what define each DNA sequencing technology, each has its own strengths and weaknesses. Comparison of important parameters between various second- and third-generation DNA sequencing technologies are presented in Table 1.

Table 1. Second- and third-generation DNA sequencing platforms

Platform	Generation	Read length (bp)	Accuracy	Cost per human genome (US$)	Cost of instrument (US$)	Run time (h/Gbp)
Massively parallel pyrosequencing by synthesis (Roche/454: GS FLX Titanium Series)	Second	400–500	Q20 read length of 40 bases (99% at 400 bases and higher for prior bases)	1,000,000	500,000	75
Sequencing by synthesis (Illumina/Solexa: Genome Analyzer IIx)	Second	2×75	Base call with Q30 (>70%)	60,000	450,000	56

Bead-based massively parallel clonal ligation based sequencing (Applied Biosystems: SOLiD 3 System)	Second	100	99.94%	60,000	591,000	42
Massively parallel single-molecule sequencing by synthesis (Helicos/Stanford Univ.)	Third	30–35	99.995% at >20×coverage (raw error rate: ≤ 5%)	70,000	1,350,000	~12
Single molecule, real time sequencing by synthesis (Pacific BioSciences/Cornell Univ.)	Third	1000–1500	99.3% at 15×coverage (error rate of a single read: 15–20%)	–	–	<1
Nanopore sequencing (Oxford Nanopore Technologies/Harvard Univ.)	Third	Potentially unlimited?	--	--	--	>20
Transmission electron microscopy single-molecule sequencing (ZS Genetics, Halcyon Molecular)	**Third**	**Potentially unlimited?**	**--**	**~10,000**	**~1,000,000**	**~14**

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Chapter 10

ATP Test, Antibiogram, Aseptic Technique and Axenic

ATP test

The **ATP test** is a process of rapidly measuring actively growing microorganisms through detection of adenosine triphosphate, or ATP.

ATP testing method

ATP is a molecule found in and around living cells, and as such it gives a direct measure of biological concentration and health. ATP is quantified by measuring the light produced through its reaction with the naturally-occurring firefly enzyme luciferase using a luminometer. The amount of light produced is directly proportional to the amount of living organisms present in the sample.

ATP tests can be used to:

- Control biological treatment reactors
- Guide biocide dosing programs
- Determine drinking water cleanliness
- Manage fermentation processes
- Assess soil activity
- Determine corrosion / deposit process type
- Measure equipment or product sanitation

1st generation testing *vs*. 2nd generation testing

1st generation ATP tests are derived from hygiene monitoring uses where samples are relatively free of interferences. 2nd Generation tests are specifically designed for water, wastewater and industrial applications where, for the most part, samples contain a variety of components that can interfere with the ATP assay.

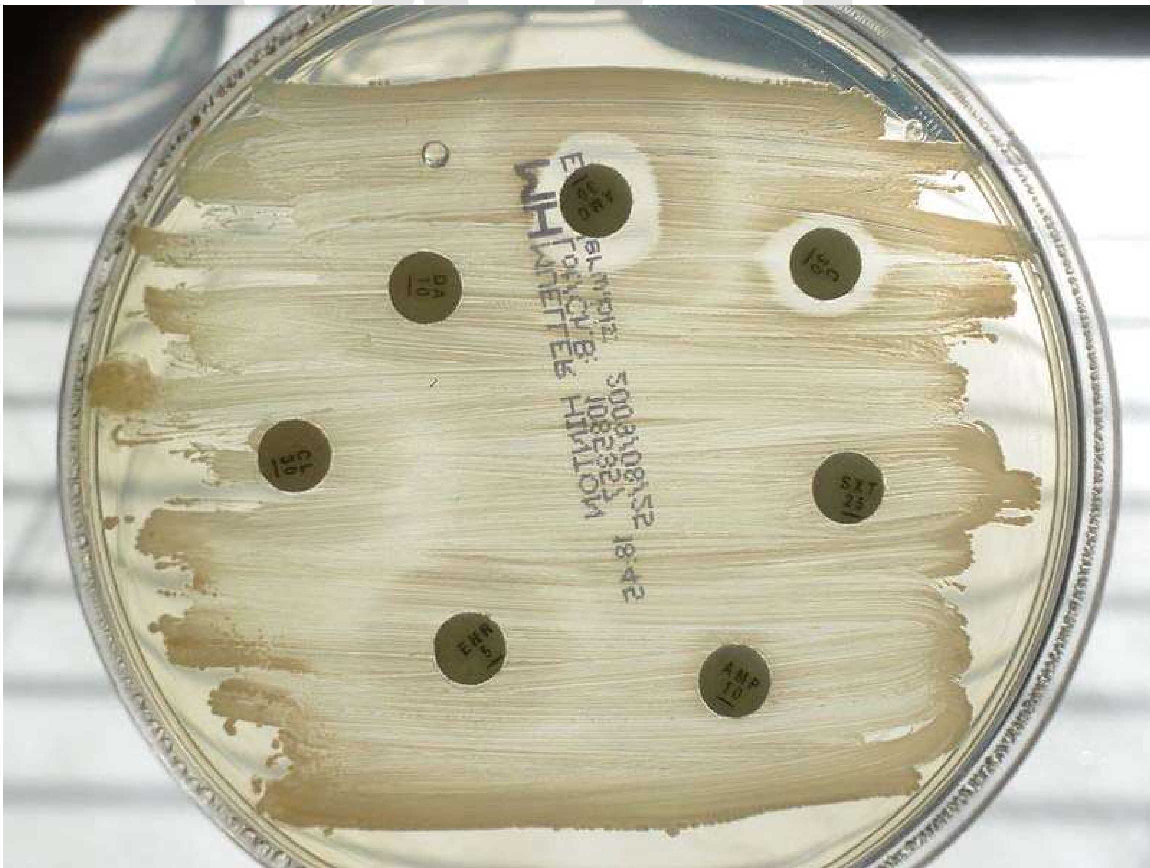
How ATP is measured

Within a water sample containing microorganisms, there are two types of ATP:

- Intracellular ATP – ATP contained within living biological cells.
- Extracellular ATP – ATP located outside of biological cells that has been released from dead or stressed organisms.

Accurate measurement of these two types of ATP is critical to utilizing ATP-based measurements. Being able to accurately measure these different types of ATP offers the ability to assess biological health and activity, and subsequently control water and wastewater processes.

Antibiogram



Antibiogram, palatine tonsil smear of a dog with tonsillitis, Mueller-Hinton agar. Only Amoxicilline-Clavulanic acid (AMC) and Chloramphenicol (C) show an inhibition of bacterial growth.

An **antibiogram** is the result of a laboratory testing for the sensitivity of an isolated bacterial strain to different antibiotics. It is by definition an *in vitro*-sensitivity.

In clinical practice, antibiotics are most frequently prescribed on the basis of general guidelines and knowledge about sensitivity: e.g. uncomplicated urinary tract infections can be treated with a first generation quinolone, etc. This is because *Escherichia coli* is the most likely causative pathogen, and it is known to be sensitive to quinolone treatment. Infections that are not acquired in the hospital, are called "community acquired" infections.

However, many bacteria are known to be resistant to several classes of antibiotics, and treatment is not so straight-forward. This is especially the case in vulnerable patients, such as patients in the intensive care unit. When these patients develop a "hospital-acquired" (or "nosocomial") pneumonia, more hardy bacteria like *Pseudomonas aeruginosa* are potentially involved. Treatment is then generally started on the basis of surveillance data about the local pathogens probably involved. This first treatment, based on statistical information about former patients, and aimed at a large group of potentially involved microbes, is called "empirical treatment".

Before starting this treatment, the physician will collect a sample from a suspected contaminated compartment: a blood sample when bacteria possibly have invaded the bloodstream, a sputum sample in the case of a ventilator associated pneumonia, and a urine sample in the case of a urinary tract infection. These samples are transferred to the microbiology lab, which looks at the sample under the microscope, and tries to culture the bacteria. This can help in the diagnosis.

Once a culture is established, there are two possible ways to get an antibiogram:

- a semi-quantitative way based on **diffusion** (Kirby-Bauer method); small discs containing different antibiotics, or impregnated paper discs, are dropped in different zones of the culture on an agar plate, which is a nutrient-rich environment in which bacteria can grow. The antibiotic will diffuse in the area surrounding each tablet, and a disc of bacterial lysis will become visible. Since the concentration of the antibiotic was the highest at the centre, and the lowest at the edge of this zone, the diameter is suggestive for the Minimum Inhibitory Concentration, or MIC, (conversion of the diameter in millimeter to the MIC, in µg/ml, is based on known linear regression curves).
- a quantitative way based on **dilution**: a dilution series of antibiotics is established (this is a series of reaction vials with progressively lower concentrations of antibiotic substance). The last vial in which no bacteria grow contains the antibiotic at the Minimal Inhibiting Concentration.

Once the MIC is calculated, it can be compared to known values for a given bacterium and antibiotic: e.g. a MIC > 0,06 µg/ml may be interpreted as a penicillin-resistant *Streptococcus pneumoniae*. Such information may be useful to the clinician, who can

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change the empirical treatment, to a more custom-tailored treatment that is directed only at the causative bacterium.

Aseptic technique

Aseptic technique refers to a procedure that is performed under sterile conditions. This includes medical and laboratory techniques, such as with microbiological cultures. It includes techniques like flame sterilization. The largest example of aseptic techniques is in hospital operating theatres.

Medical procedures

Aseptic technique is the effort taken to keep patients as free from hospital micro-organisms as possible (Crow 1989). It is a method used to prevent contamination of wounds and other susceptible sites by organisms that could cause infection. This can be achieved by ensuring that only sterile equipment and fluids are used during invasive medical and nursing procedures. Ayliffe et al. (2000) suggest that there are two types of asepsis: medical and surgical asepsis. Medical or clean asepsis reduces the number of organisms and prevents their spread; surgical or sterile asepsis includes procedures to eliminate micro-organisms from an area and is practiced by surgical technologists and nurses in operating theaters and treatment areas. In an operating room, while all members of the surgical team should demonstrate good aseptic technique, it is the role of the scrub nurse or surgical technologist to set up and maintain the sterile field.

Axenic

Physcomitrella patens plants growing axenically on an agar plate (Petri dish, 9 cm diameter).

In biology, **axenic** describes a culture of an organism that is entirely free of all other "contaminating" organisms. The earliest axenic cultures were of bacteria or unicellular eukaryotes, but axenic cultures of many multicellular organisms are also possible. Axenic culture is an important tool for the study of symbiotic and parasitic organisms in a controlled manner.

Preparation

Axenic cultures of microorganisms are typically prepared using a dilution series of an existing mixed culture. This culture is successively diluted to the point where subsamples of it contain only a few individual organisms, ideally only a single individual (in the case of an asexual species). These subcultures are allowed to grow until the identity of their constituent organisms can be ascertained. Selection of those cultures consisting solely of the desired organism produces the axenic culture.

Axenic cultures are usually checked routinely to ensure that they remain axenic. One standard approach with microorganisms is to spread a sample of the culture onto an agar plate, and to incubate this for a fixed period of time. The agar should be an enriched medium that will support the growth of common "contaminating" organisms. Such "contaminating" organisms will grow on the plate during this period, identifying cultures that are no longer axenic.

Experimental use

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As axenic cultures are derived from very few organisms, or even a single individual, they are useful because the organisms present within them share a relatively narrow gene pool. In the case of an asexual species derived from a single individual, the resulting culture should consist of identical organisms (though processes such as mutation and horizontal gene transfer may introduce a degree of variability). Consequently, they will generally respond in a more uniform and reproducible fashion, simplifying the interpretation of experiments.

Problems

The axenic culture of some pathogens is complicated because they normally thrive within host tissues which exhibit properties that are difficult to replicate *in vitro*. This is especially true in the case of intracellular pathogens. However, careful replication of key features of the host environment can resolve these difficulties (e.g. host metabolites, dissolved oxygen), such as with the Q fever pathogen, *Coxiella burnetii*.

Chapter 11

Bacteriological Water Analysis and Clonogenic Assay

Bacteriological water analysis

Bacteriological water analysis is a method of analysing water to estimate the numbers of bacteria present and, if needed, to find out what sort of bacteria they are. It is a microbiological analytical procedure which uses samples of water and from these samples determines the concentration of bacteria. It is then possible to draw inferences about the suitability of the water for use from these concentrations. This process is used, for example, to routinely confirm that water is safe for human consumption or that bathing and recreational waters are safe to use.

The interpretation and the action trigger levels for different waters vary depending on the use made of the water. Very stringent levels applying to drinking water whilst more relaxed levels apply to marine bathing waters where much lower volumes of water are expected to be ingested by users.

Approach

The common feature of all these routine screening procedures is that the primary analysis is for indicator organisms rather than the pathogens that might cause concern. Indicator organisms are bacteria such as non-specific coliforms, *Escherichia coli* and *Pseudomonas aeruginosa* that are very commonly found in the human or animal gut and which, if detected, may suggest the presence of sewage. Indicator organisms are used because even when a person is infected with a more pathogenic bacteria, they will still be excreting many millions times more indicator organisms than pathogens. It is therefore reasonable to surmise that if indicator organism levels are low, then pathogen levels will be very much lower or absent. Judgements as to suitability of water for use are based on very extensive precedents and relate to the probability of any sample population of bacteria being able to be infective at a reasonable statistical level of confidence.

Analysis is usually performed using culture, biochemical and sometimes optical methods. When indicator organisms levels exceed pre-set triggers, specific analysis for pathogens may then be undertaken and these can be quickly detected (where suspected) using specific culture methods or molecular biology.

Methodologies

Because the analysis is always based on a very small sample taken from a very large volume of water, all methods rely on statistical principles.

Multiple tube method

One of the oldest methods is called the multiple tube method. In this method a measured sub-sample (perhaps 10ml) is diluted with 100ml of sterile growth medium and an aliquot of 10ml is then decanted into each of ten tubes. The remaining 10ml is then diluted again and the process repeated. At the end of 5 dilutions this produces 50 tubes covering the dilution range of 1:10 through to 1: 10000. The tubes are then incubated at a pre-set temperature for a specified time and at the end of the process the number of tubes with growth in is counted for each dilution. Statistical tables are then used to derive the concentration of organisms in the original sample. This method can be enhanced by using indicator medium which changes colour when acid forming species are present and by including a tiny inverted tube in each sample tube. This inverted tube catches any gas produced. The production of gas at 37 Deg Celsius is a strong indication of the presence of *Escherichia coli*

ATP Testing

An ATP test is the process of rapidly measuring active microorganisms in water through detection of a molecule called Adenosine Triphosphate, or ATP.

ATP is a molecule found only in and around living cells, and as such it gives a direct measure of biological concentration and health. ATP is quantified by measuring the light produced through its reaction with the naturally-occurring firefly enzyme Luciferase using a Luminometer. The amount of light produced is directly proportional to the amount of biological energy present in the sample

2nd Generation ATP tests are specifically designed for water, wastewater and industrial applications where, for the most part, samples contain a variety of components that can interfere with the ATP assay.

Plate count

The plate count method relies on bacteria growing a colony on a nutrient medium so that the colony becomes visible to the naked eye and the number of colonies on a plate can be counted. To be effective, the dilution of the original sample must be arranged so that on

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average between 30 and 300 colonies of the target bacterium are grown. Fewer than 30 colonies makes the interpretation statistically unsound whilst greater than 300 colonies often results in overlapping colonies and imprecision in the count. To ensure that an appropriate number of colonies will be generated several dilutions are normally cultured.

The laboratory procedure involves making serial dilutions of the sample (1:10, 1:100, 1:1000 etc.) in sterile water and cultivating these on nutrient agar in a dish that is sealed and incubated. Typical media include Plate count agar for a general count or MacConkey agar to count gram-negative bacteria such as *E. coli*. Typically one set of plates is incubated at 22°C and for 24 hours and a second set at 37°C for 24 hours. The composition of the nutrient usually includes reagents that resist the growth of non-target organisms and make the target organism easily identified, often by a colour change in the medium. Some recent methods include a fluorescent agent so that counting of the colonies can be automated. At the end of the incubation period the colonies are counted by eye, a procedure that takes a few moments and does not require a microscope as the colonies are typically a few millimetres across.

Membrane filtration

Most modern laboratories use a refinement of total plate count in which serial dilutions of the sample are vacuum filtered through purpose made membrane filters and these filters are themselves laid on nutrient medium within sealed plates. The methodology is otherwise similar to conventional total plate counts. Membranes have a printed millimetre grid printed on and can be reliably count a much greater number of colonies under a binocular microscope.

Pour plates

When the analysis is looking for bacterial species that grow poorly in air, the initial analysis is done by mixing serial dilutions of the sample in liquid nutrient agar which is then poured into bottles which are then sealed and laid on their sides to produce a sloping agar surface. Colonies that develop in the body of the medium can be counted by eye after incubation.

The total number of colonies is referred to as the Total Viable Count (TVC). The unit of measurement is cfu/ml (or colony forming units per millilitre) and relates to the original sample. Calculation of this is a multiple of the counted number of colonies multiplied by the dilution used.

Pathogen analysis

When samples show elevated levels of indicator bacteria, further analysis is often undertaken to look for specific pathogenic bacteria. Species commonly investigated in the temperate zone include *Salmonella typhi* and *Salmonella typhimurium* Depending on the likely source of contamination investigation may also extend to organisms such as

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Cryptosporidium spp. In tropical areas analysis of *Vibrio cholerae* is also routinely undertaken.

Types of nutrient media used in analysis

MacConkey agar is culture medium designed to grow Gram-negative bacteria and stain them for lactose fermentation. It contains bile salts (to inhibit most Gram-positive bacteria), crystal violet dye (which also inhibits certain Gram-positive bacteria), neutral red dye (which stains microbes fermenting lactose), lactose and peptone. Alfred Theodore MacConkey developed it while working as a bacteriologist for the Royal Commission on Sewage Disposal in the United Kingdom.

ENDO medium contains peptone, lactose, dipotassium phosphate, agar, sodium sulfite, basic fuchsin and was originally developed for the isolation of *Salmonella typhi*, but is now commonly used in water analysis. As in MacConkey agar, coliform organisms ferment the lactose, and the colonies become red. Non-lactose-fermenting organisms produce clear, colourless colonies against the faint pink background of the medium.

mFC medium is a medium used in membrane filtration which contains selective and differential agents. These include Rosolic acid to inhibit bacterial growth in general, except for faecal coliforms, Bile salts inhibit non-enteric bacteria and Aniline blue indicates the ability of faecal coliforms to ferment lactose to acid that causes a pH change in the medium.

TYEA medium contains tryptone, yeast extract, common salt and L-arabinose per liter of glass distilled water and is a non selective medium usually cultivated at two temperatures (22 and 36°C) to determine a general level of contamination (a.k.a colony count).

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Clonogenic assay

A **clonogenic assay** is a microbiology technique for studying the effectiveness of specific agents on the survival and proliferation of cells. It is frequently used in cancer research laboratories to determine the effect of drugs or radiation on proliferating tumor cells as well as for titration of Cell-killing Particles (CKP) in virus stocks.

Although this technique can provide accurate results, the assay is time-consuming to set up and analyse and can only provide data on tumor cells that can grow in culture. The word "clonogenic" refers to the fact that these cells are clones of one another.

Procedure

The experiment involves three major steps:

1. The treatment is applied to a sample of cells.
2. The cells are "plated" in a tissue culture vessel and allowed to grow.
3. The colonies produced are fixed, stained, and counted.

At the conclusion of the experiment, the percentage of cells that survived the treatment is measured. A graphical representation of survival versus drug concentration or dose of ionizing radiation is called a *cell survival curve*.

For Cell-killing Particle assays, the surviving fraction of cells is used to approximate the Poisson Distribution of virus particles amongst cells and therefore determine the number of CKP encountered by each cell.

Any type of cell could be used in an experiment, but since the goal of these experiments in oncological research is the discovery of more effective cancer treatments, human tumor cells are a typical choice. The cells either come from prepared "cell lines," which have been well-studied and whose general characteristics are known, or from a biopsy of a tumor in a patient. The cells are put in petri dishes or in plates which contain several circular "wells." Particular numbers of cells are plated depending on the experiment; for an experiment involving irradiation it is usual to plate larger numbers of cells with increasing dose of radiation. For example, at a dose of 0 or 1 gray of radiation, 500 cells might be plated, but at 4 or 5 gray, 2500 might be plated, since very large numbers of cells are killed at this level of radiation and the effects of the specific treatment would be unobservable.

Counting the cell colonies is usually done under a microscope and is quite tedious. Recently, machines have been developed that use algorithms to analyse images. These are either captured by a image scanner or an automated microscope that can completely automate the counting process. One such automated machine works by accepting certain types of cell plates through a slot (not unlike a CD player), taking a photograph, and uploading it to a computer for immediate analysis. Reliable counts are available in seconds. As of 2004, these machines are expensive, with basic models retailing for over USD$30,000.

Variables

The treatment is usually a drug, ionizing radiation, or a combination of the two. Some current research studies the potentiation of drug effects by concurrent irradiation—a synergistic effect—and in this situation two groups are studied: a control group, which is not treated with the drug; and a treatment group, which is treated with the drug. Both groups are irradiated. If the slopes of their survival curves differ significantly, then a potentiating effect may be evident and could be studied further.

A thorough discussion of the promising research being conducted with the aid of this technique is beyond the scope of this text, but some studies involve the effect of the expression of particular genes or receptors on the cell, the responses of different cell types, or synergistic effects of multiple drugs.

Chapter 12

Gentamicin Protection Assay, Hydrodynamic Focusing and Industrial Fermentation

Gentamicin protection assay

The **gentamicin protection assay** or **survival assay** or **invasion assay** is a method used in microbiology. It is used to quantify the ability of pathogenic bacteria to invade eukaryotic cells.

The assay is based on several observations made in the 1970s, in which the ability of internalized bacteria to avoid killing by antibiotics was reported. The assay started to be used in biological research in the early 1980s.

Background and principle

Intracellular bacteria need to enter host cells (cells of the infected organism) in order to replicate and propagate infection. Many species of *Shigella* (causes bacillary dysentery), *Salmonella* (typhoid fever), *Mycobacterium* (leprosy and tuberculosis) and *Listeria* (listeriosis), to name but a few, are intracellular.

Several antibiotics cannot penetrate eukaryotic cells. Therefore, these antibiotics cannot hurt intracellular bacteria that are already internalized. Using such antibiotics enables us to differentiate between bacteria that succeed in penetrating eukaryotic cells and those that do not. Applying such an antibiotic to a culture of eukaryotic cells infected with bacteria would kill the bacteria that remain outside the cells while sparing the ones that penetrated. The antibiotic of choice for this assay is the aminoglycoside gentamicin.

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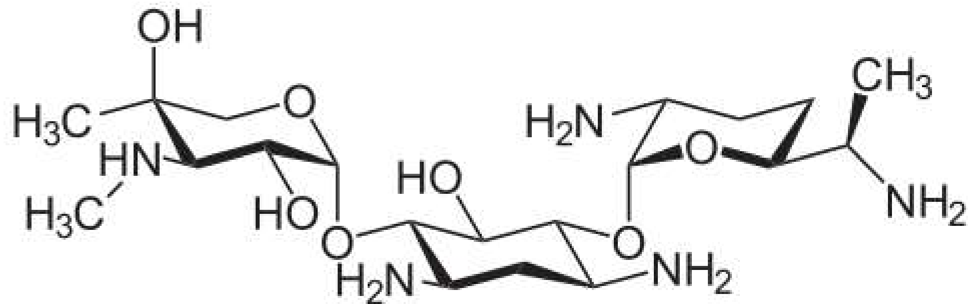
Procedure



The chemical structure of gentamicin

HeLa cells are commonly used as eukaryotic cells in the gentamicin protection assay, but other cells can be used as well. As for bacteria, only species susceptible to gentamicin can be assayed.

The assay is performed in polypropylene plates with round wells, which are commonly used in laboratories for culturing eukaryotic cells. The cells are allowed to grow in the wells overnight, creating a flat layer. Bacteria are separately grown overnight. On the next day the eukaryotic cells are inoculated with the bacteria and are incubated together for an hour. Centrifuging the plates for a few minutes may help bring cells and bacteria in contact and initiate infection.

After infection gentamicin is added to the plates, and they are incubated for an hour, allowing the antibiotic to kill all bacteria that were not able to penetrate the cells and remained outside. The plates are then washed well to remove the dead bacteria. Next the eukaryotic cells are lysed using a detergent, most commonly Triton X-100.

The bacteria that penetrated the cells and remained alive are now released, and they are plated on solid medium plates. Counting the colonies formed on the plates on the next day, and knowing how many bacteria were used in the beginning of the assay, enables the researcher to calculate the percentage of bacteria that were able to invade the eukaryotic cells.

Usage, advantages and caveats

The gentamicin protection assay is commonly used in pathogen research. The contribution of specific genes or proteins to the bacteria's ability to invade cells can be easily assayed using this method. The gene in question can be knocked out, and the bacteria's invasiveness compared with that of normal, wild type bacteria. Environmental conditions, such as pH level and temperature, can also be assayed for their effect on invasiveness.

The gentamicin protection assay is very sensitive, as it can detect the internalization of even single bacteria. It has several drawbacks:

- Gentamicin can sometimes penetrate eukaryotic cells and kill the internalized bacteria. This may happen if the permeability of the cells somehow increased during the assay, sometimes due to poor handling of the cells.
- Internalized bacteria may sometimes not be entirely protected from the outside environment, such as when the phagosome (the vacuole surrounding the bacterium inside the cell) is defective in some way. Gentamicin may kill those bacteria.
- Gentamicin may fail to kill all the bacteria that remained outside the cells.

To help assess the accuracy of a particular assay, positive and negative controls should be performed. When performing the assay as described above, bacteria that are known to be entirely invasive (positive control) and bacteria that are known as non-invasive (negative control) should be included in the assay.

An alternative invasion assay is the differential immunostaining assay, based on the binding of antibodies to bacteria before and after invasion. The antibodies emit fluorescent, colored light, and the results of this assay are viewed under the microscope.

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Hydrodynamic focusing

Hydrodynamic focusing is a technique used by microbiologists to provide more accurate results from flow cytometers or Coulter counters for determining the size of bacteria or cells.

Measuring particles

Counting cells happens by forcing them to pass through a small tunnel, causing disruptions in a laser light beam or electricity flow. These disruptions are being analyzed by the instruments. It is hard to create these small tunnels for these cells using ordinary manufacturing processes, as the diameter should be on the magnitude of micrometers, and the length of the tunnel should exceed several millimeters.

Focusing with a fluid

Hydrodynamic focusing solves this by building up the walls of the tunnel from fluid, using the effects of fluid dynamics. There is a wide (hundreds of micrometers in diameter) tube created of glass or plastic, and a "wall" fluid called the sheath fluid is being pumped through. The sample is injected into the middle of the sheath flow. If the two fluids differ enough in their velocity or density.

Industrial fermentation

Industrial fermentation is the intentional use of fermentation by microorganisms such as bacteria and fungi to make products useful to humans. Fermented products have applications as food as well as in general industry.

Food fermentation

Ancient fermented food processes, such as making bread, wine, cheese, curds, idli, dosa, etc., can be dated to more than 6,000 years ago. They were developed long before man had any knowledge of the existence of the microorganisms involved. Fermentation is also a powerful economic incentive for semi-industrialized countries, in their willingness to produce bio-ethanol.

Pharmaceuticals and the biotechnology industry

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There are 5 major groups of commercially important fermentation:

1. Microbial cells or biomass as the product, e.g. single cell protein, bakers yeast, lactobacillus, E. coli, etc.
2. Microbial enzymes: catalase, amylase, protease, pectinase, glucose isomerase, cellulase, hemicellulase, lipase, lactase, streptokinase, etc.
3. Microbial metabolites :
    1. Primary metabolites – ethanol, citric acid, glutamic acid, lysine, vitamins, polysaccharides etc.
    2. Secondary metabolites: all antibiotic fermentation
4. Recombinant products: insulin, HBV, interferon, GCSF, streptokinase
5. Biotransformations: phenyl acetyl carbinol, steroid biotransformation, etc.

Nutrient sources for industrial fermentation

Growth media are required for industrial fermentation, since any microbe requires water, (oxygen), an energy source, a carbon source, a nitrogen source and micronutrients for growth.

Carbon & energy source + nitrogen source + O_2 + other requirements → Biomass + Product + byproducts + CO_2 + H_2O + heat

Nutrient	Raw material
	Carbon
Glucose	corn sugar, starch, cellulose
Sucrose	sugarcane, sugar beet molasses
glycerol	

Starch	
Maltodextrine	
Lactose	milk whey
fats	vegetable oils
Hydrocarbons	petroleum fractions
Nitrogen	
Protein	soybean meal, corn steep liquor, distillers' solubles
Ammonia	pure ammonia or ammonium salts urea
Nitrate	nitrate salts
Phosphorus source	phosphate salts
Vitamins and growth factors	
	Yeast, Yeast extract
	Wheat germ meal, cotton seed meal
	Beef extract
	Corn steep liquor

Trace elements: Fe, Zn, Cu, Mn, Mo, Co

Antifoaming agents : Esters, fatty acids, fats, silicones, sulphonates, polypropylene glycol

Buffers: Calcium carbonate, phosphates

Growth factors: Some microorganisms cannot synthesize the required cell components themselves and need to be supplemented, e.g. with thiamine, biotin, calcium pentothenate

Precursors: Directly incorporated into the desired product: Phenyl ethylamine into Benzyl penicillin, Phenyl acetic acid into Penicillin G

Inhibitors: To get the specific products: e.g. sodium barbital for rifamycin

Inducers: The majority of the enzymes used in industrial fermentation are inducible and are synthesized in response of inducers: e.g. starch for amylases, maltose for pollulanase, pectin for pectinase,olive oil and tween are also used at times.

Chelators: Chelators are the chemicals used to avoid the precipitation of metal ions. Chelators like EDTA, citric acid, polyphosphates are used in low concentrations.

Sewage disposal

In the process of sewage disposal, sewage is digested by enzymes secreted by bacteria. Solid organic matters are broken down into harmless, soluble substances and carbon

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dioxide. Liquids that result are disinfected to remove pathogens before being discharged into rivers or the sea or can be used as liquid fertilisers. Digested solids, known also as sludge, is dried and used as fertilisers. Gaseous by-products such as methane, can be utilised as biogas to fuel generators. One advantage of bacterial digestion is that it reduces the bulk and odour of sewage, thus reducing space needed for dumping, on the other hand, a major disadvantage of bacterial digestion in sewage disposal is that it is a very slow process.

Phases of microbial growth

When a particular organism is introduced into a selected growth medium, the medium is inoculated with the particular organism. Growth of the inoculum does not occur immediately, but takes a little while. This is the period of adaptation, called the lag phase. Following the lag phase, the rate of growth of the organism steadily increases, for a certain period--this period is the log or exponential phase. After a certain time of exponential phase, the rate of growth slows down, due to the continuously falling concentrations of nutrients and/or a continuously increasing (accumulating) concentrations of toxic substances. This phase, where the increase of the rate of growth is checked, is the deceleration phase. After the deceleration phase, growth ceases and the culture enters a stationary phase or a steady state. The biomass remains constant, except when certain accumulated chemicals in the culture lyse the cells (chemolysis). Unless other micro-organisms contaminate the culture, the chemical constitution remains unchanged. Mutation of the organism in the culture can also be a source of contamination, called internal contamination.

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Chapter 13

Microscopy

Microscopy is the technical field of using microscopes to view samples and objects that cannot be seen with the unaided eye (objects that are not within the resolution range of the normal eye). There are three well-known branches of microscopy, optical, electron, and scanning probe microscopy.

Optical and electron microscopy involve the diffraction, reflection, or refraction of electromagnetic radiation/electron beams interacting with the specimen, and the subsequent collection of this scattered radiation or another signal in order to create an image. This process may be carried out by wide-field irradiation of the sample (for example standard light microscopy and transmission electron microscopy) or by scanning of a fine beam over the sample (for example confocal laser scanning microscopy and scanning electron microscopy). Scanning probe microscopy involves the interaction of a scanning probe with the surface of the object of interest. The development of microscopy revolutionized biology and remains an essential technique in the life and physical sciences.

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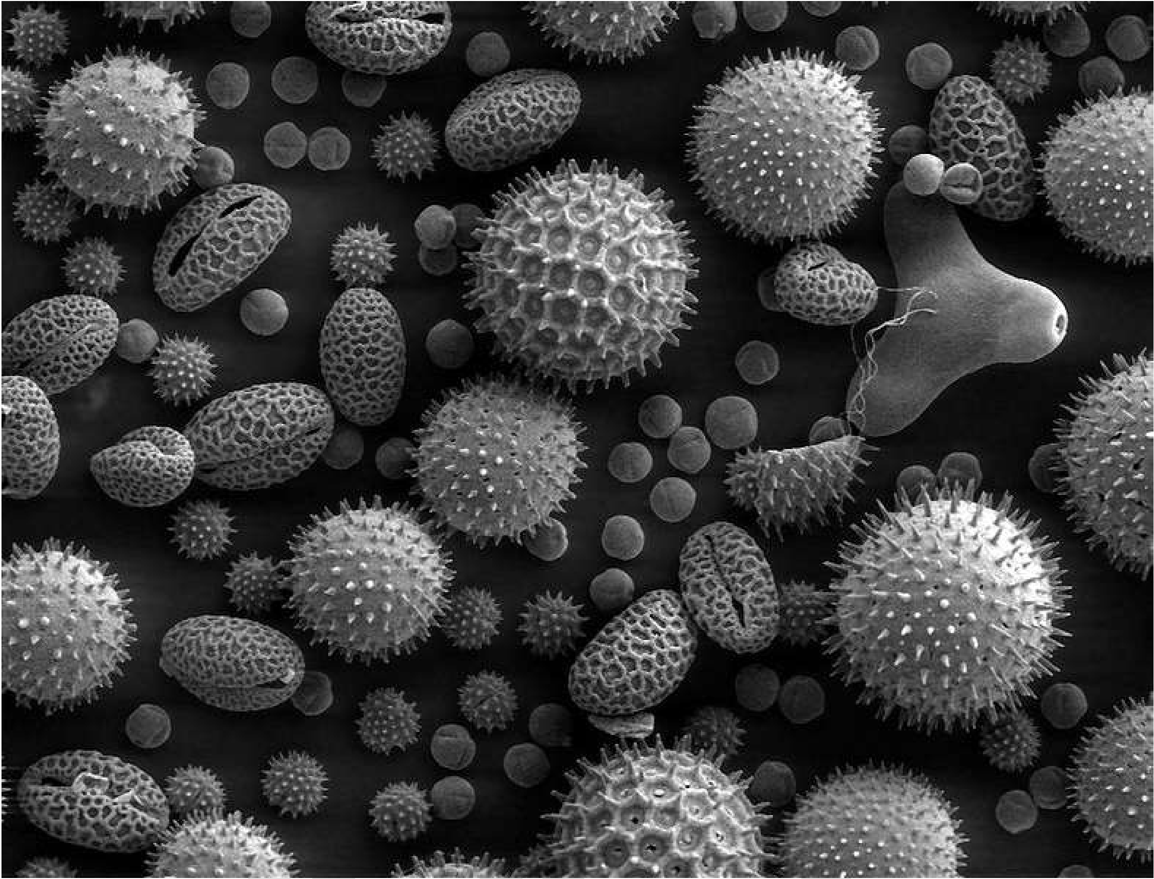

Scanning electron microscope image of pollen.

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Optical microscopy

Stereo microscope

Optical or light microscopy involves passing visible light transmitted through or reflected from the sample through a single or multiple lenses to allow a magnified view of the sample. The resulting image can be detected directly by the eye, imaged on a photographic plate or captured digitally. The single lens with its attachments, or the system of lenses and imaging equipment, along with the appropriate lighting equipment, sample stage and support, makes up the basic light microscope. The most recent

development is the digital microscope, which uses a CCD camera to focus on the exhibit of interest. The image is shown on a computer screen, so eye-pieces are unnecessary.

Limitations

Limitations of standard optical microscopy (bright field microscopy) lie in three areas;

- The technique can only image dark or strongly refracting objects effectively.
- Diffraction limits resolution to approximately 0.2 micrometre (*see: microscope*).
- Out of focus light from points outside the focal plane reduces image clarity.

Live cells in particular generally lack sufficient contrast to be studied successfully, internal structures of the cell are colourless and transparent. The most common way to increase contrast is to stain the different structures with selective dyes, but this involves killing and fixing the sample. Staining may also introduce artifacts, apparent structural details that are caused by the processing of the specimen and are thus not a legitimate feature of the specimen.

These limitations have all been overcome to some extent by specific microscopy techniques that can non-invasively increase the contrast of the image. In general, these techniques make use of differences in the refractive index of cell structures. It is comparable to looking through a glass window: you (bright field microscopy) don't see the glass but merely the dirt on the glass. There is however a difference as glass is a denser material, and this creates a difference in phase of the light passing through. The human eye is not sensitive to this difference in phase but clever optical solutions have been thought out to change this difference in phase into a difference in amplitude (light intensity).

Techniques

In order to improve specimen contrast or highlight certain structures in a sample special techniques must be used. A huge selection of microscopy techniques are available to increase contrast or label a sample.

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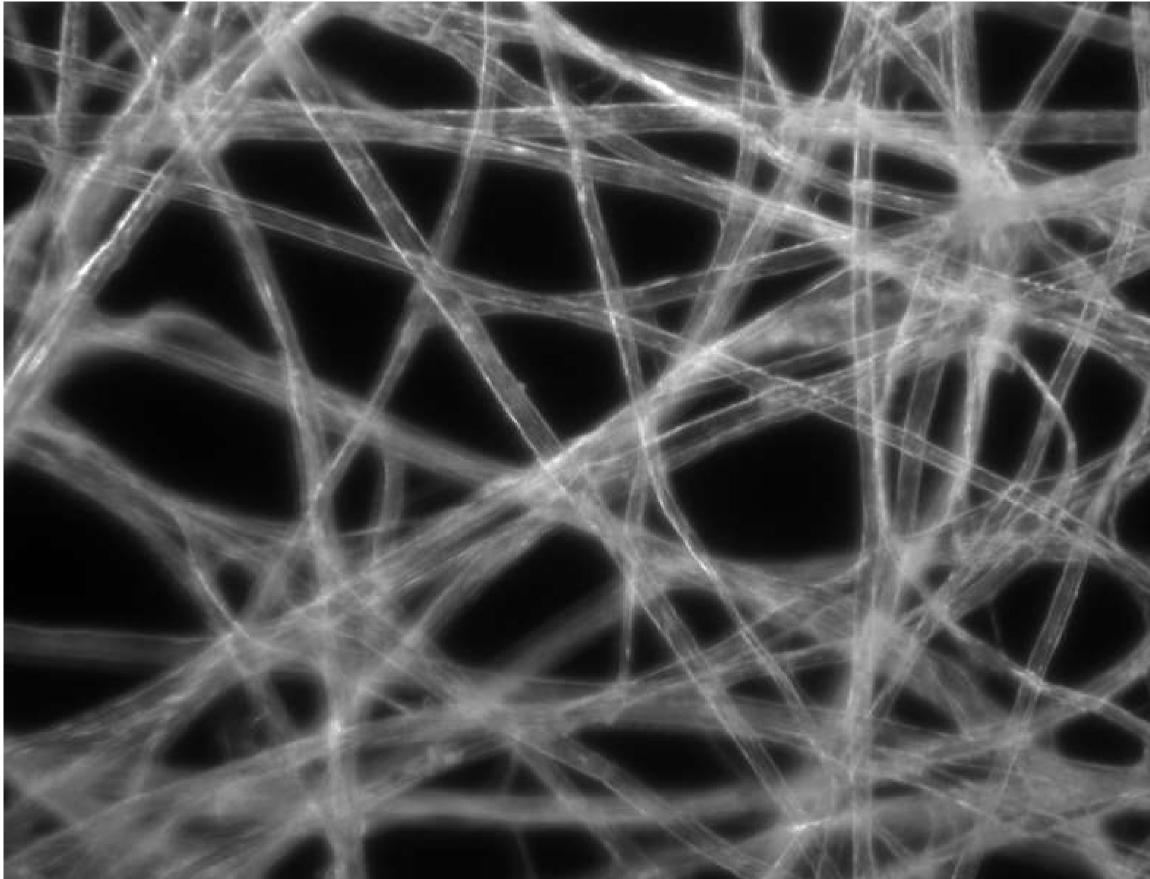
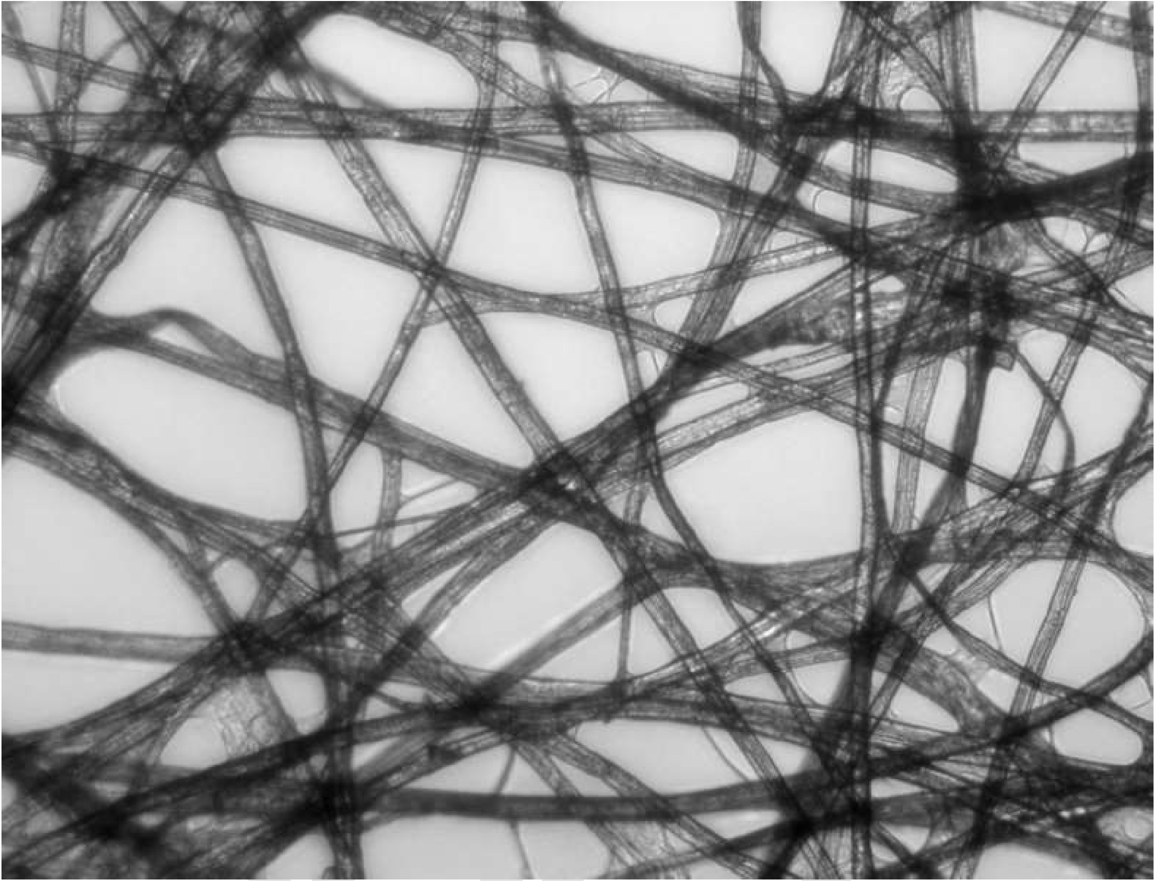
Comparison of transilumination techniques used to generate contrast in a sample of tissue paper. 1.559 μm/pixel.



Dark field illumination, sample contrast comes from light scattered by the sample.



Bright field illumination, sample contrast comes from absorbance of light in the sample.

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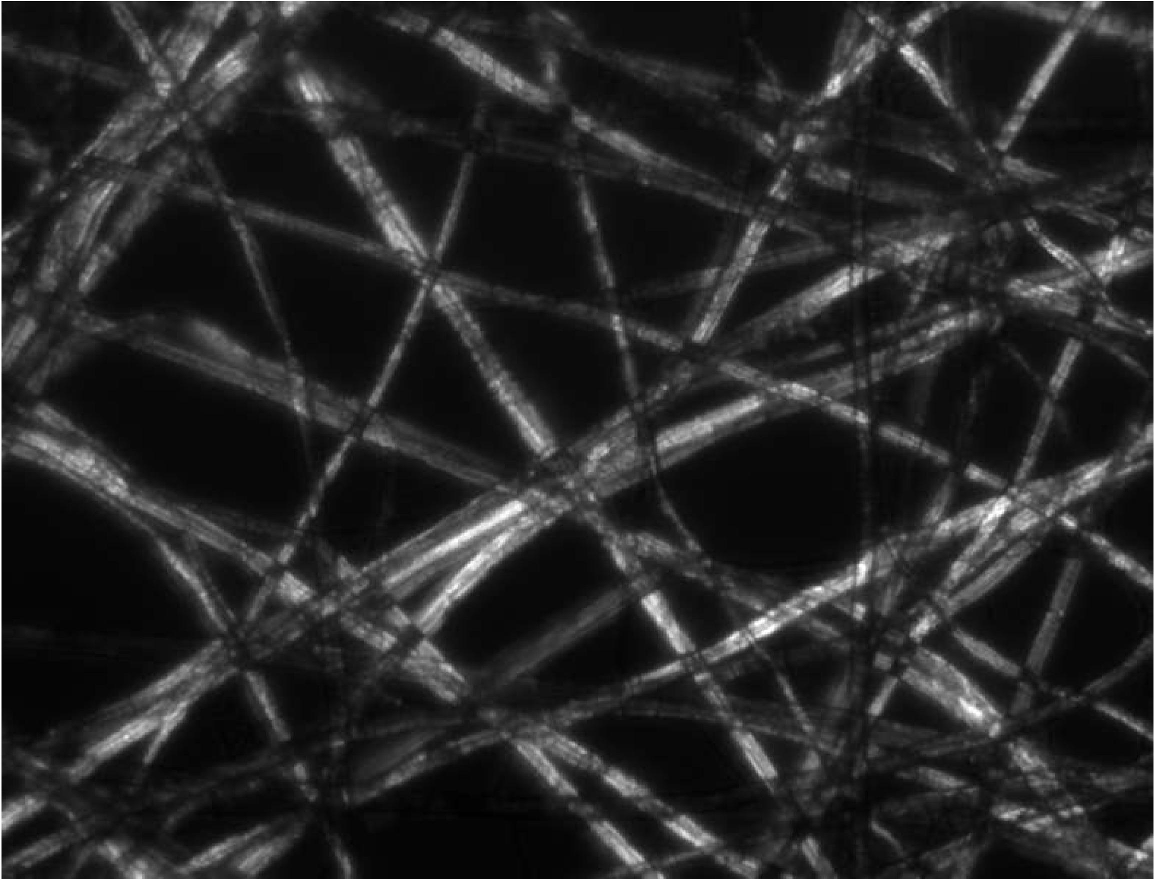

Cross-polarized light illumination, sample contrast comes from rotation of polarized light through the sample.

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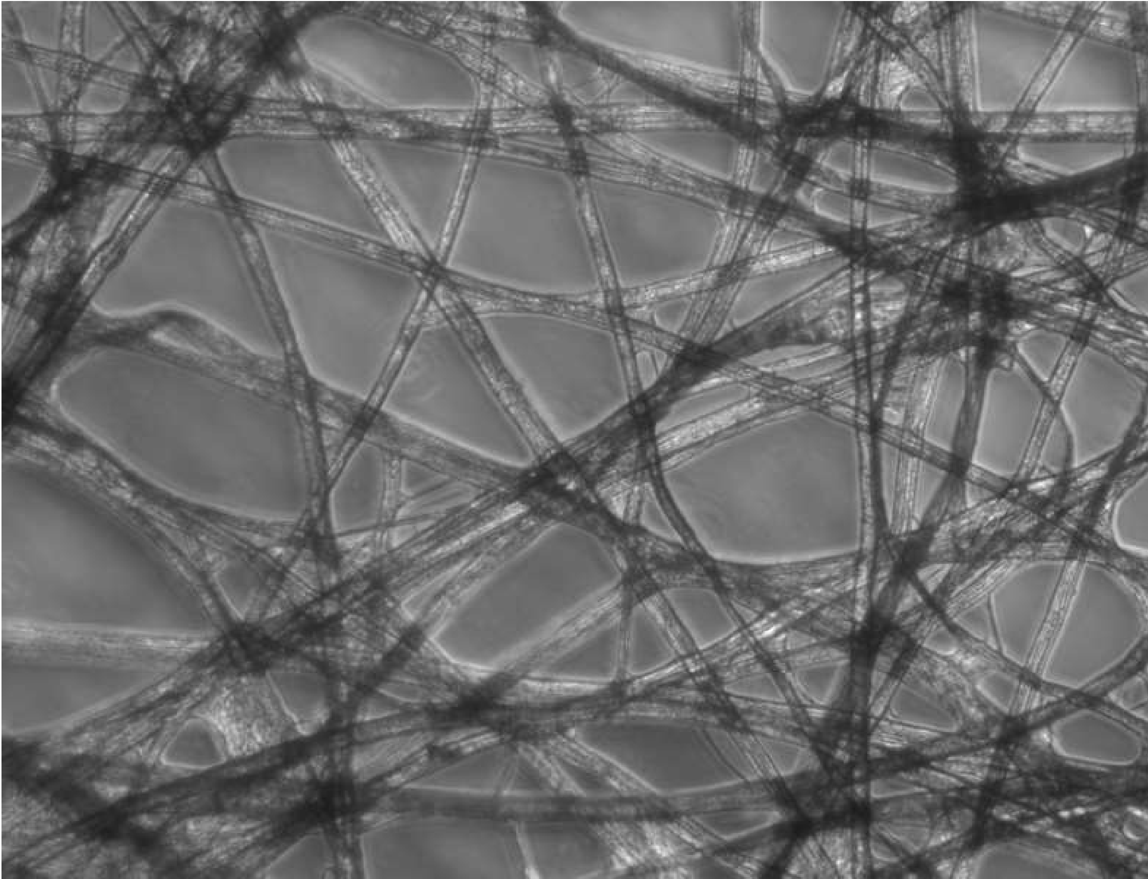

Phase contrast illumination, sample contrast comes from interference of different path lengths of light through the sample.

Bright field

Bright field microscopy is the simplest of all the light microscopy techniques. Sample illumination is via transmitted white light, i.e. illuminated from below and observed from above. Limitations include low contrast of most biological samples and low apparent resolution due to the blur of out of focus material. The simplicity of the technique and the minimal sample preparation required are significant advantages.

Oblique illumination

The use of oblique (from the side) illumination gives the image a 3-dimensional appearance and can highlight otherwise invisible features. A more recent technique based on this method is *Hoffmann's modulation contrast*, a system found on inverted microscopes for use in cell culture. Oblique illumination suffers from the same limitations as bright field microscopy (low contrast of many biological samples; low apparent resolution due to out of focus objects), but may highlight otherwise invisible structures.

Dark field

Dark field microscopy is a technique for improving the contrast of unstained, transparent specimens. Dark field illumination uses a carefully aligned light source to minimize the quantity of directly-transmitted (unscattered) light entering the image plane, collecting only the light scattered by the sample. Darkfield can dramatically improve image contrast—especially of transparent objects – while requiring little equipment setup or sample preparation. However, the technique does suffer from low light intensity in final image of many biological samples, and continues to be affected by low apparent resolution.

Rheinberg illumination is a special variant of dark field illumination in which transparent, colored filters are inserted just before the condenser so that light rays at high aperture are differently colored than those at low aperture (i.e. the background to the specimen may be blue while the object appears self-luminous yellow). Other color combinations are possible but their effectiveness is quite variable.

Dispersion staining

Dispersion staining is an optical technique that results in a colored image of a colorless object. This is an optical staining technique and requires no stains or dyes to produce a color effect. There are five different microscope configurations used in the broader technique of dispersion staining. They include brightfield Becke` line, oblique, darkfield, phase contrast, and objective stop dispersion staining.

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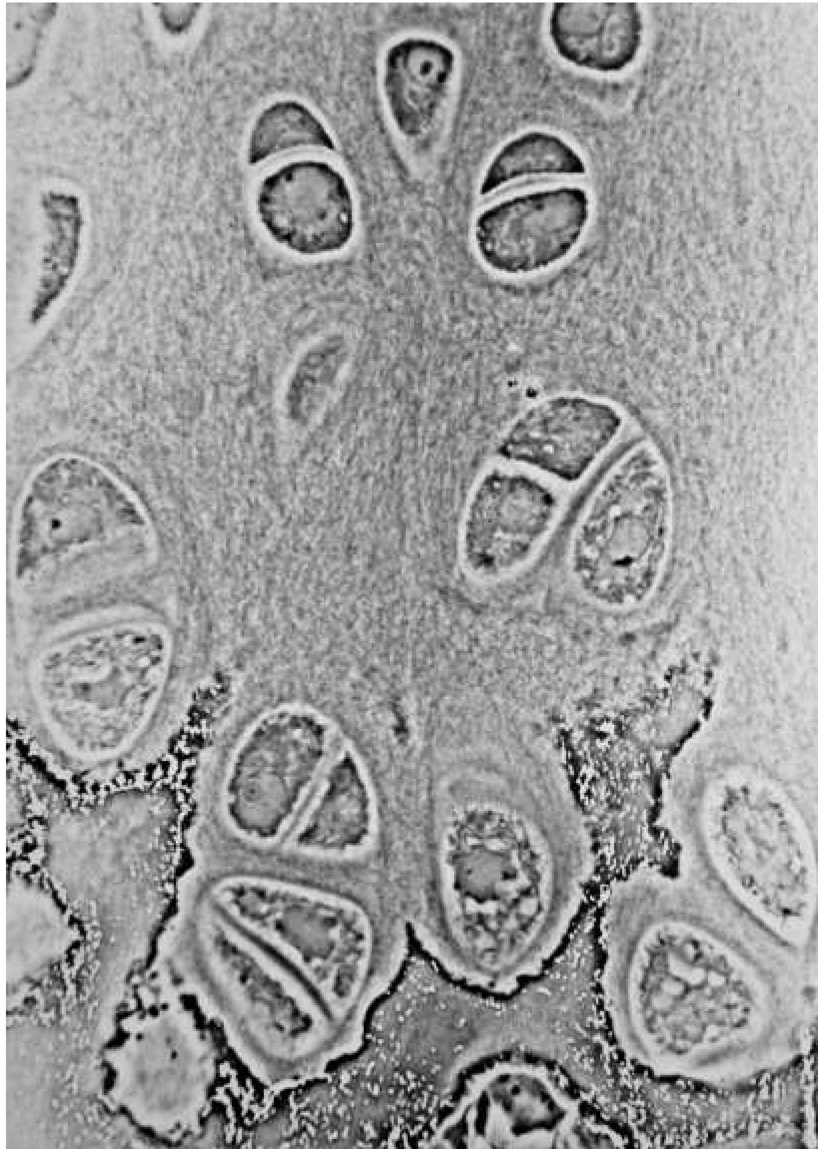
Phase contrast



Phase-contrast image of uncalcified matrix (top) and calcified matrix (bottom).

More sophisticated techniques will show proportional differences in optical density . **Phase contrast** is a widely used technique that shows differences in refractive index as difference in contrast. It was developed by the Dutch physicist Frits Zernike in the 1930s (for which he was awarded the Nobel Prize in 1953). The nucleus in a cell for example will show up darkly against the surrounding cytoplasm. Contrast is excellent; however it is not for use with thick objects. Frequently, a halo is formed even around small objects, which obscures detail. The system consists of a circular annulus in the condenser, which produces a cone of light. This cone is superimposed on a similar sized ring within the phase-objective. Every objective has a different size ring, so for every objective another condenser setting has to be chosen. The ring in the objective has special optical

properties: it first of all reduces the direct light in intensity, but more importantly, it creates an artificial phase difference of about a quarter wavelength. As the physical properties of this direct light have changed, interference with the diffracted light occurs, resulting in the phase contrast image.

one disadvantge of phasecontrast microscopy is halo formation (halo-light ring)

Differential interference contrast

Superior and much more expensive is the use of **interference contrast**. Differences in optical density will show up as differences in relief. A nucleus within a cell will actually show up as a globule in the most often used **differential interference contrast** system according to Georges Nomarski. However, it has to be kept in mind that this is an *optical effect*, and the relief does not necessarily resemble the true shape! Contrast is very good and the condenser aperture can be used fully open, thereby reducing the depth of field and maximizing resolution.

The system consists of a special prism (Nomarski prism, Wollaston prism) in the condenser that splits light in an ordinary and an extraordinary beam. The spatial difference between the two beams is minimal (less than the maximum resolution of the objective). After passage through the specimen, the beams are reunited by a similar prism in the objective.

In a homogeneous specimen, there is no difference between the two beams, and no contrast is being generated. However, near a refractive boundary (say a nucleus within the cytoplasm), the difference between the ordinary and the extraordinary beam will generate a relief in the image. Differential interference contrast requires a polarized light source to function; two polarizing filters have to be fitted in the light path, one below the condenser (the polarizer), and the other above the objective (the analyzer).

Note: In cases where the optical design of a microscope produces an appreciable lateral separation of the two beams we have the case of classical interference microscopy, which does not result in relief images, but can nevertheless be used for the quantitative determination of mass-thicknesses of microscopic objects.

Interference reflection microscopy

An additional technique using interference is **interference reflection microscopy** (also known as reflected interference contrast, or RIC). It is used to examine the adhesion of cells to a glass surface, using polarized light of a narrow range of wavelengths to be reflected whenever there is an interface between two substances with different refractive indices. Whenever a cell is attached to the glass surface, reflected light from the glass and from the attached cell will interfere, while if there is no cell attached to the glass, there will be no interference.

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Interference reflection microscopy can be obtained by using the same elements used by DIC, but without the prisms. Also, the light that is being detected is reflected and not transmitted as it is when DIC is employed.

Fluorescence

When certain compounds are illuminated with high energy light, they then emit light of a different, lower frequency. This effect is known as fluorescence. Often specimens show their own characteristic autofluorescence image, based on their chemical makeup.

This method is of critical importance in the modern life sciences, as it can be extremely sensitive, allowing the detection of single molecules. Many different fluorescent dyes can be used to stain different structures or chemical compounds. One particularly powerful method is the combination of antibodies coupled to a fluorophore as in immunostaining. Examples of commonly used fluorophores are fluorescein or rhodamine. The antibodies can be made tailored specifically for a chemical compound. For example, one strategy often in use is the artificial production of proteins, based on the genetic code (DNA). These proteins can then be used to immunize rabbits, which then form antibodies which bind to the protein. The antibodies are then coupled chemically to a fluorophore and then used to trace the proteins in the cells under study.

Highly-efficient fluorescent proteins such as the green fluorescent protein (GFP) have been developed using the molecular biology technique of gene fusion, a process that links the expression of the fluorescent compound to that of the target protein. This combined fluorescent protein is, in general, non-toxic to the organism and rarely interferes with the function of the protein under study. Genetically modified cells or organisms directly express the fluorescently-tagged proteins, which enables the study of the function of the original protein in vivo.

Since fluorescence emission differs in wavelength (color) from the excitation light, an ideal fluorescent image shows only the structure of interest that was labeled with the fluorescent dye. This high specificity led to the widespread use of fluorescence light microscopy in biomedical research. Different fluorescent dyes can be used to stain different biological structures, which can then be detected simultaneously, while still being specific due to the individual color of the dye.

To block the excitation light from reaching the observer or the detector, filter sets of high quality are needed. These typically consist of an excitation filter selecting the range of excitation wavelengths, a dichroic mirror, and an emission filter blocking the excitation light. Most fluorescence microscopes are operated in the Epi-illumination mode (illumination and detection from one side of the sample) to further decrease the amount of excitation light entering the detector.

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Confocal

Using a scanning point of light instead of full sample illumination confocal microscopy gives slightly higher resolution, and significant improvements in optical sectioning . Confocal microscopy is, therefore, commonly used where 3D structure is important.

Deconvolution

Fluorescence microscopy is extremely powerful due to its ability to show specifically labeled structures within a complex environment and also because of its inherent ability to provide three-dimensional information of biological structures. However, this information is blurred by the fact that, upon illumination, all fluorescently labeled structures emit light no matter whether they are in focus or not. This means that an image of a certain structure is always blurred by the contribution of light from structures that are out of focus. This phenomenon becomes apparent as a loss of contrast especially when using objectives with a high resolving power, typically oil immersion objectives with a high numerical aperture.

However, this phenomenon is not caused by random processes such as light scattering but can be relatively well defined by the optical properties of the image formation in the microscope imaging system. If one considers a small fluorescent light source (essentially a bright spot), light coming from this spot spreads out the further out of focus one is. Under ideal conditions, this produces a sort of "hourglass" shape of this point source in the third (axial) dimension. This shape is called the point spread function (PSF) of the microscope imaging system. Since any fluorescence image is made up of a large number of such small fluorescent light sources, the image is said to be "convolved by the point spread function".

Knowing this point spread function means that it is possible to reverse this process to a certain extent by computer-based methods commonly known as deconvolution microscopy. There are various algorithms available for 2D or 3D deconvolution. They can be roughly classified in *nonrestorative* and *restorative* methods. While the nonrestorative methods can improve contrast by removing out-of-focus light from focal planes, only the restorative methods can actually reassign light to its proper place of origin. This can be an advantage over other types of 3D microscopy such as confocal microscopy, because light is not thrown away but reused. For 3D deconvolution, one typically provides a series of images derived from different focal planes (called a Z-stack) plus the knowledge of the PSF, which can be derived either experimentally or theoretically from knowing all contributing parameters of the microscope.

Sub-diffraction techniques

It is well known that there is a spatial limit to which light can focus: approximately half of the wavelength of the light one is using. But this is not a true barrier, because this diffraction limit is only true in the far-field and localization precision can be increased with many photons and careful analysis; and like the sound barrier, the diffraction barrier

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is breakable. Here we, explores some approaches to imaging objects smaller than ~250 nm. In 1978, the first theoretical ideas had been developed to break this barrier using a 4Pi microscope as a confocal laser scanning fluorescence microscope where the light is focused ideally from all sides to a common focus that is used to scan the object by 'point-by-point' excitation combined with 'point-by-point' detection. Most of the following information was gathered (with permission) from a chemistry blog's review of sub-diffraction microscopy techniques Part I and Part II.

Vertico SMI - SPDMphymod Superresolution Microscopy

Localization Microscopy/Spatially Structured Illumination

Around 1995, Christoph Cremer commenced with the development of a light microscopic process, which achieved a substantially improved size resolution of cellular nanostructures stained with a fluorescent marker. This time he employed the principle of wide field microscopy combined with structured laser illumination (spatially modulated illumination, SMI. In addition, this technology is no longer subjected to the speed limitations of the focusing microscopy so that it becomes possible to undertake 3D analyses of whole cells within short observation times (at the moment around a few seconds).

Also since around 1995, Christoph Cremer developed and realized new fluorescence-based wide-field microscopy approaches that had as their goal the improvement of the effective optical resolution (in terms of the smallest detectable distance between two localized objects) down to a fraction of the conventional resolution (spectral precision distance/position determination microscopy, SPDM).

Combining SPDM and SMI, known as Vertico SMI microscopy Christoph Cremer can currently achieve a resolution of approx. 10 nm in 2D and 40 nm in 3D in wide field images of whole living cells. The Vertico SMI is currently the fastest optical 3D nanoscope for the three-dimensional structural analysis of whole cells world-wide.

The Vertico SMI works with high recording speed and processes a complete 3D stack in 40 seconds (2000 frames: 50 frames/s), the very fast image processing based on specific proprietary algorithms makes the image available after 2min/3min (1-/2-color). The specific wide-field technique captures very large areas up to 5000 µm2.

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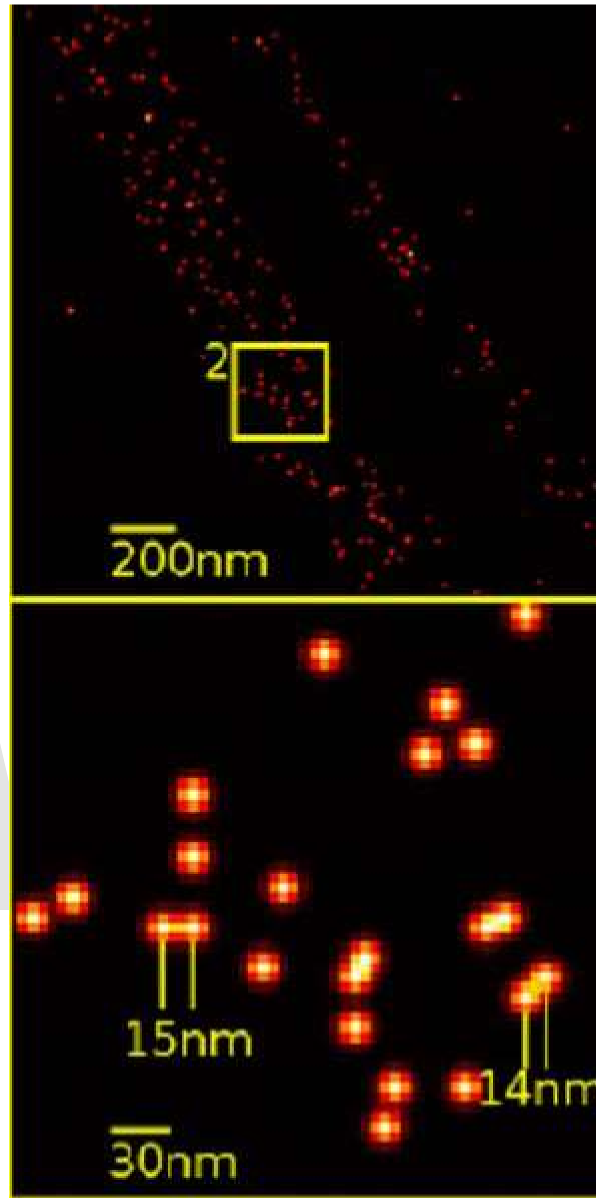
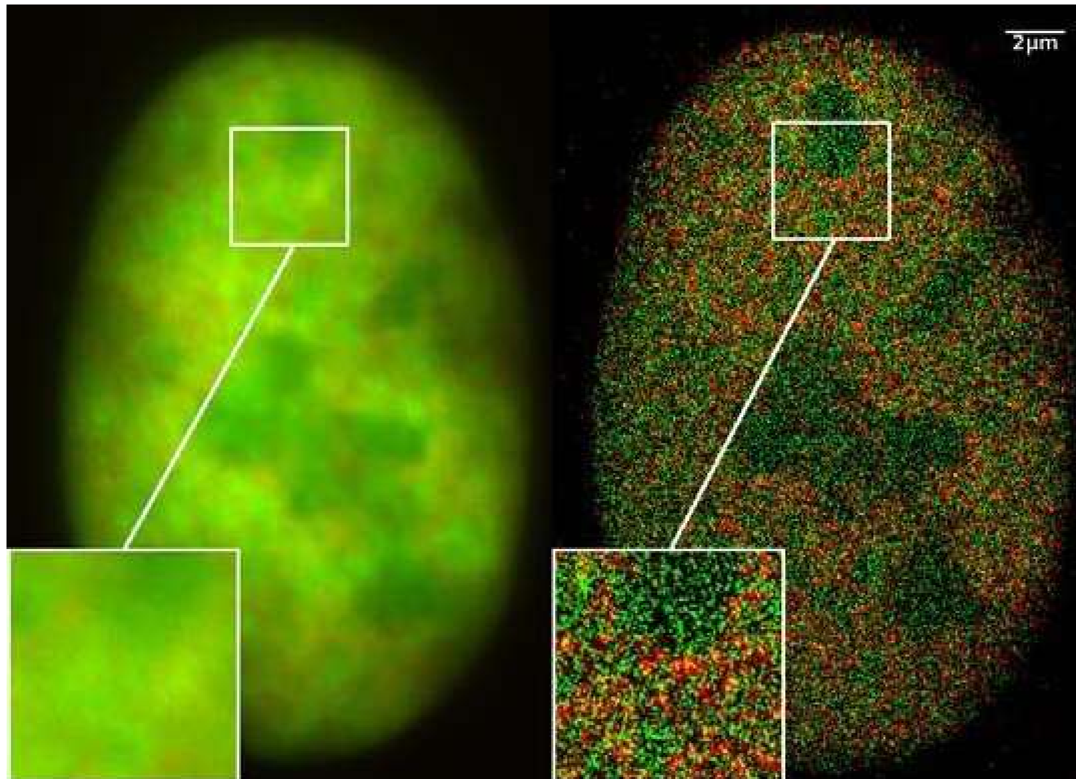
SPDMphymod: Super Resolution Microscopy Images of standard GFP, RFP, YFP fluorescent dyes



Single YFP molecule detection in a human cancer cell. Typical distance measurements in the 15 nm range (5 nm standard deviation)



Co-localisation microscopy (2CLM) with GFP and RFP fusion proteins (nucleus of a bone cancer cell) 120.000 localized molecules in a widefield area (470 µm2)

Use of standard dyes like normal GFP

In 2008, Cremer's lab discovered that super resolution microscopy was also possible for many standard fluorescent dyes like GFP, Alexa dyes and fluorescein molecules, provided certain photo-physical conditions are present. Using his specific localization microscopy called SPDMPhymod, it is possible to detect and count two different fluorescent molecule types (this technology is referred to as 2CLM, 2 Color Localization Microscopy)

Near-field scanning

Near-field scanning is also called NSOM. Probably the most conceptual way to break the diffraction barrier is to use a light source and/or a detector that is itself nanometer in scale. Diffraction as we know it is truly a far-field effect: The light from an aperture is the Fourier transform of the aperture in the far-field. But, in the near-field, all of this is not necessarily the case. Near-field scanning optical microscopy (NSOM) forces light through the tiny tip of a pulled fiber — and the aperture can be on the order of tens of nanometers. When the tip is brought to nanometers away from a molecule, the resolution is limited not by diffraction but by the size of the tip aperture (because only that one molecule will see the light coming out of the tip). An image can be built by a raster scan of the tip over the surface to create an image.

The main down-side to NSOM is the limited number of photons you can force out a tiny tip, and the minuscule collection efficiency (if one is trying to collect fluorescence in the near-field). Other techniques such as ANSOM try to avoid this drawback.

Local enhancement / ANSOM / optical nano-antennas

Instead of forcing photons down a tiny tip, some techniques create a local bright spot in an otherwise diffraction-limited spot. ANSOM is apertureless NSOM: it uses a tip very close to a fluorophore to enhance the local electric field the fluorophore sees. Basically, the ANSOM tip is like a lightning rod which creates a hot spot of light.

Bowtie nanoantennas have been used to greatly and reproducibly enhance the electric field in the nanometer gap between the tips two gold triangles. Again, the point is to enhance a very small region of a diffraction-limited spot, thus improving the mismatch between light and nanoscale objects—and breaking the diffraction barrier.

Stimulated emission depletion

Stefan Hell at the Max Planck Institute for Biophysical Chemistry - Göttingen (Germany) developed STED microscopy (stimulated emission depletion), which uses two laser pulses. The first pulse is a diffraction-limited spot that is tuned to the absorption wavelength, so excites any fluorophores in that region; an immediate second pulse is red-shifted to the emission wavelength and stimulates emission back to the ground state before, thereby depleting the excited state of any fluorophores in this depletion pulse. The trick is that the depletion pulse goes through a phase modulator that makes the pulse illuminate the sample in the shape of a donut, *so the outer part of the diffraction limited spot is depleted and the small center can still fluoresce*. By saturating the depletion pulse, the center of the donut gets smaller and smaller until they can get resolution of tens of nanometers.

This technique also requires a raster scan like NSOM and standard confocal laser scanning microscopy.

Fitting the point-spread function

Fitting the point-spread function (also called PSF). The methods above (and below) use experimental techniques to circumvent the diffraction barrier, but one can also use crafty analysis to increase the ability to know where a nanoscale object is located. The image of a point source on a charge-coupled device camera is called a point-spread function (PSF), which is limited by diffraction to be no less than approximately half the wavelength of the light. But it is possible to simply fit that PSF with a Gaussian to locate the center of the PSF — and thus the location of the fluorophore. The precision by which this technique can locate the center depends on the number of photons collected (as well as the CCD pixel size and other factors). This concept was first used to achieve resolution beyond the diffraction limit with single molecules by Van Oijen et al. in 1998 (Chem. Phys. Lett. V.292, p183). Subsequently at room temperature, groups like the Selvin lab

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and many others have employed this analysis to localize single fluorophores to a few nanometers. This, of course, requires careful measurements and collecting *many* photons.

PALM, STORM

What fitting a PSF is to localization, photo-activated localization microscopy (PALM) is to "resolution"—this term is here used loosely to mean measuring the distance between objects, not true optical resolution. Eric Betzig and colleagues developed PALM; Xiaowei Zhuang at Harvard used a similar technique and calls it STORM: stochastic optical reconstruction microscopy. Sam Hess at University of Maine developed the technique simultaneously. The basic premise of both techniques is to fill the imaging area with many dark fluorophores that can be photoactivated into a fluorescing state by a flash of light. Because photoactivation is stochastic, only a few, well-separated molecules "turn on." Then Gaussians are fit to their PSFs to high precision. After the few bright dots photobleach, another flash of the photoactivating light activates random fluorophores again and the PSFs are fit of these different well-spaced objects. This process is repeated many times, building up an image molecule-by-molecule; and, because the molecules were localized at different times, the "resolution" of the final image can be much higher than that limited by diffraction.

The major problem with these techniques is that to get these beautiful pictures, it takes on the order of hours to collect the data. This is not the technique to study dynamics (fitting the PSF is better for that).

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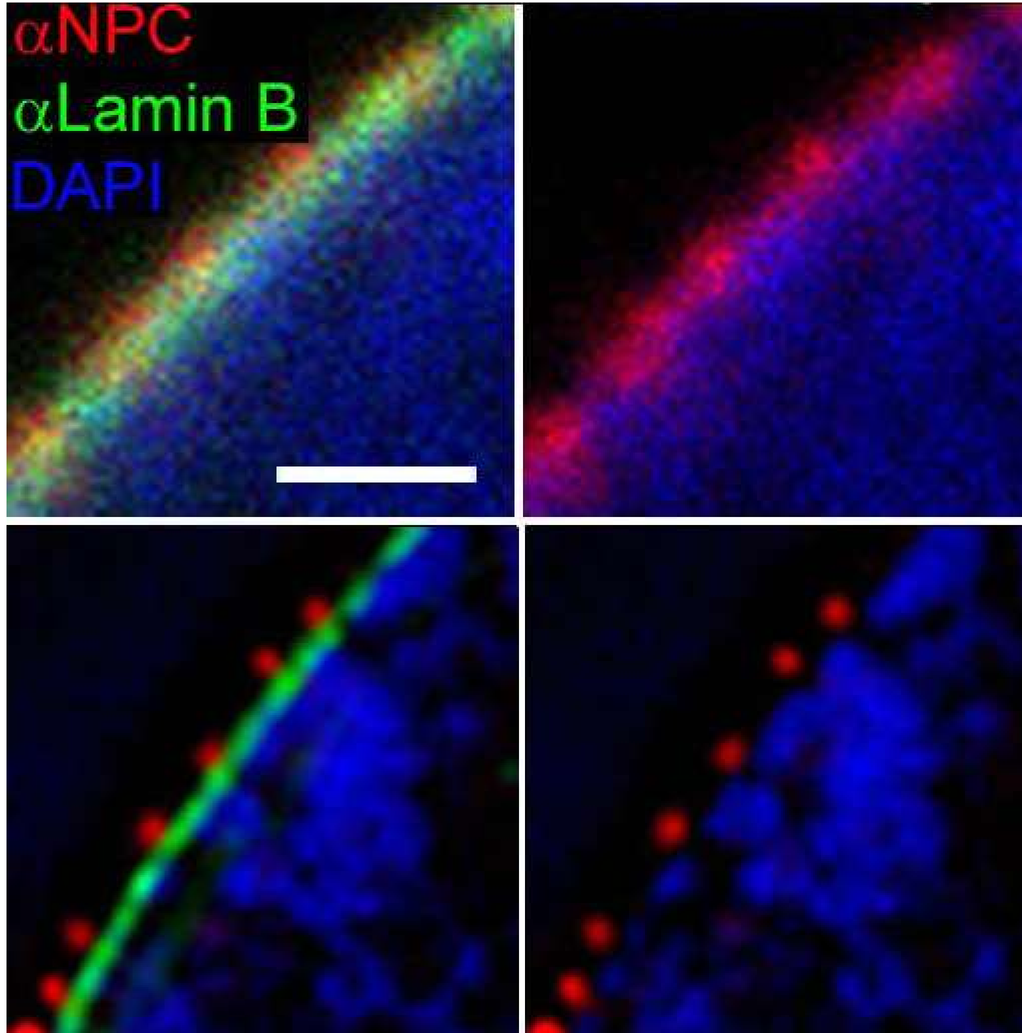
Structured illumination



Comparison of the resolution obtained by confocal laser scanning microscopy (top) and 3D structured illumination microscopy (3D-SIM-Microscopy, bottom). Shown are details of a nuclear envelope. Nuclear pores (anti-NPC) red, nuclear envelope (anti-Lamin) green, chromatin (DAPI-staining) blue. Scale bars: 1µm.

There is also the wide-field structured-illumination (SI) approach to breaking the diffraction limit of light. SI—or patterned illumination—relies on both specific microscopy protocols and extensive software analysis post-exposure. But, because SI is a wide-field technique, it is usually able to capture images at a higher rate than confocal-based schemes like STED (but SI is not actually superfast). The main concept of SI is to illuminate a sample with patterned light and increase the resolution by measuring the fringes in the Moiré pattern (from the interference of the illumination pattern and the sample). "Otherwise-unobservable sample information can be deduced from the fringes and computationally restored."

SI enhances spatial resolution by collecting information from frequency space outside the observable region. This process is done in reciprocal space: The Fourier transform (FT) of an SI image contains superimposed additional information from different areas of reciprocal space; with several frames with the illumination shifted by some phase, it is possible to computationally separate and reconstruct the FT image, which has much more resolution information. The reverse FT returns the reconstructed image to a super-resolution image.

But this enhances the resolution only by a factor of 2 (because the SI pattern cannot be focused to anything smaller than half the wavelength of the excitation light). To further increase the resolution, one can introduce *nonlinearities*, which show up as higher-order harmonics in the FT. In reference , Gustafsson uses saturation of the fluorescent sample as the nonlinear effect. A sinusoidal saturating excitation beam produces the distorted fluorescence intensity pattern in the emission. This nonpolynomial nonlinearity yields a series of higher-order harmonics in the FT.

Each higher-order harmonic in the FT allows another set of images that can be used to reconstruct a larger area in reciprocal space, and thus a higher resolution. In this case, Gustafsson achieves less than 50-nm resolving power, more than five times that of the microscope in its normal configuration.

The main problems with SI are that, in this incarnation, saturating excitation powers cause more photodamage and lower fluorophore photostability, and sample drift must be kept to below the resolving distance. The former limitation might be solved by using a different nonlinearity (such as stimulated emission depletion or reversible photoactivation, both of which are used in other sub-diffraction imaging schemes); the latter limits live-cell imaging and may require faster frame rates or the use of some fiduciary markers for drift subtraction. Nevertheless, SI is certainly a strong contender for further application in the field of super-resolution microscopy.

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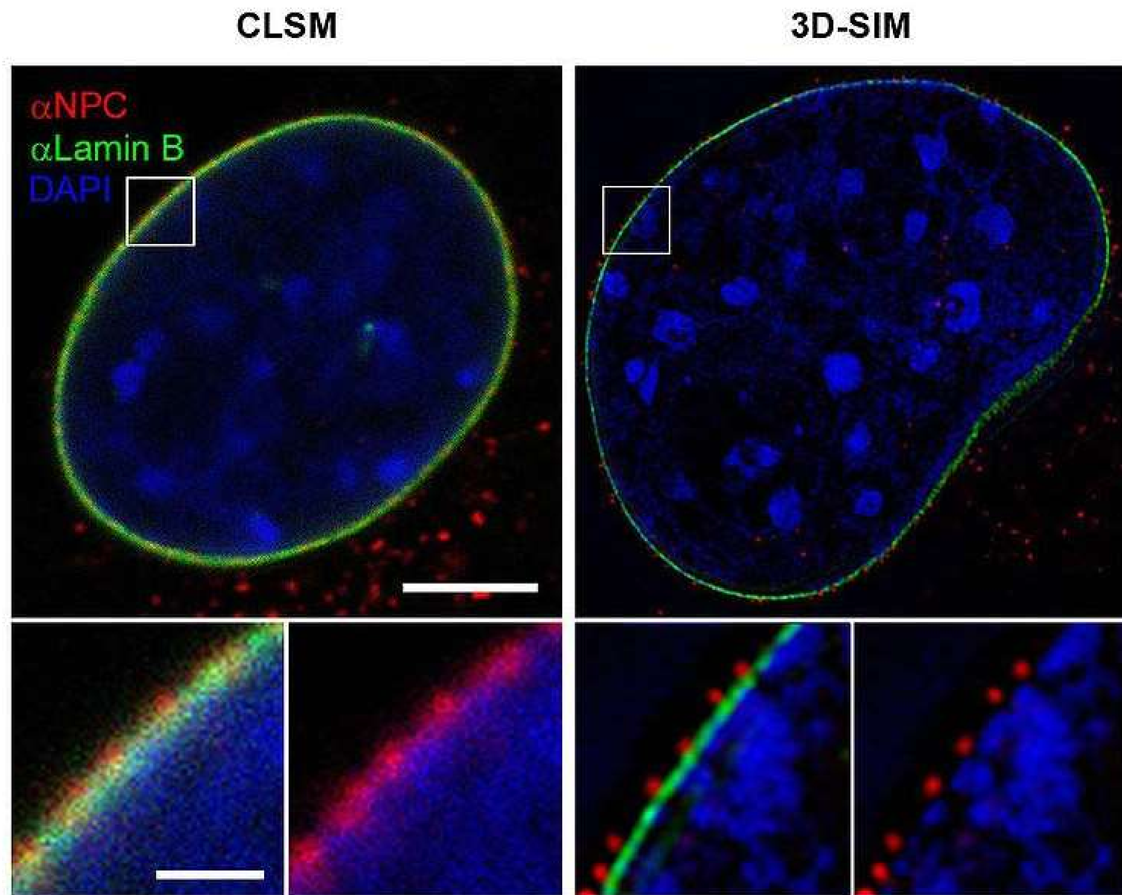
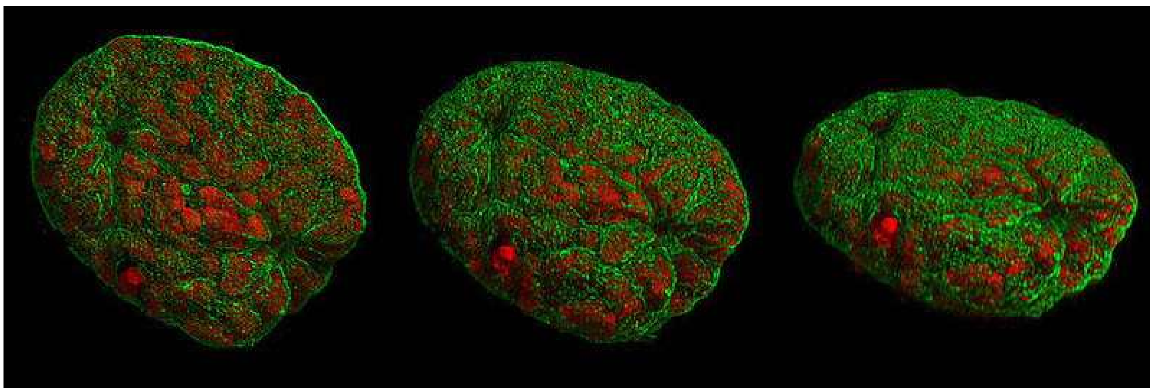
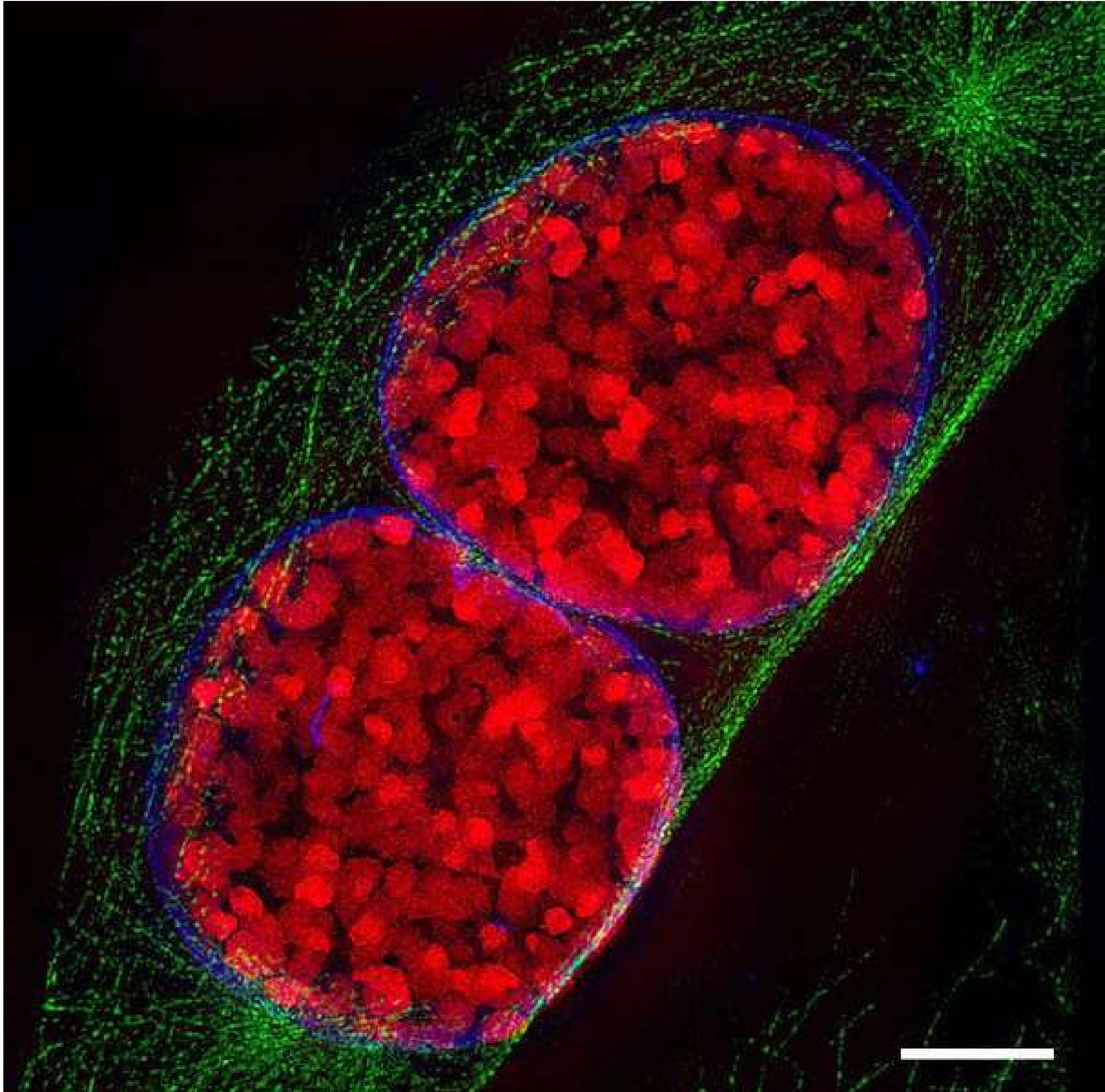
Images of cell nuclei and mitotic stages recorded with 3D-SIM Microscopy.



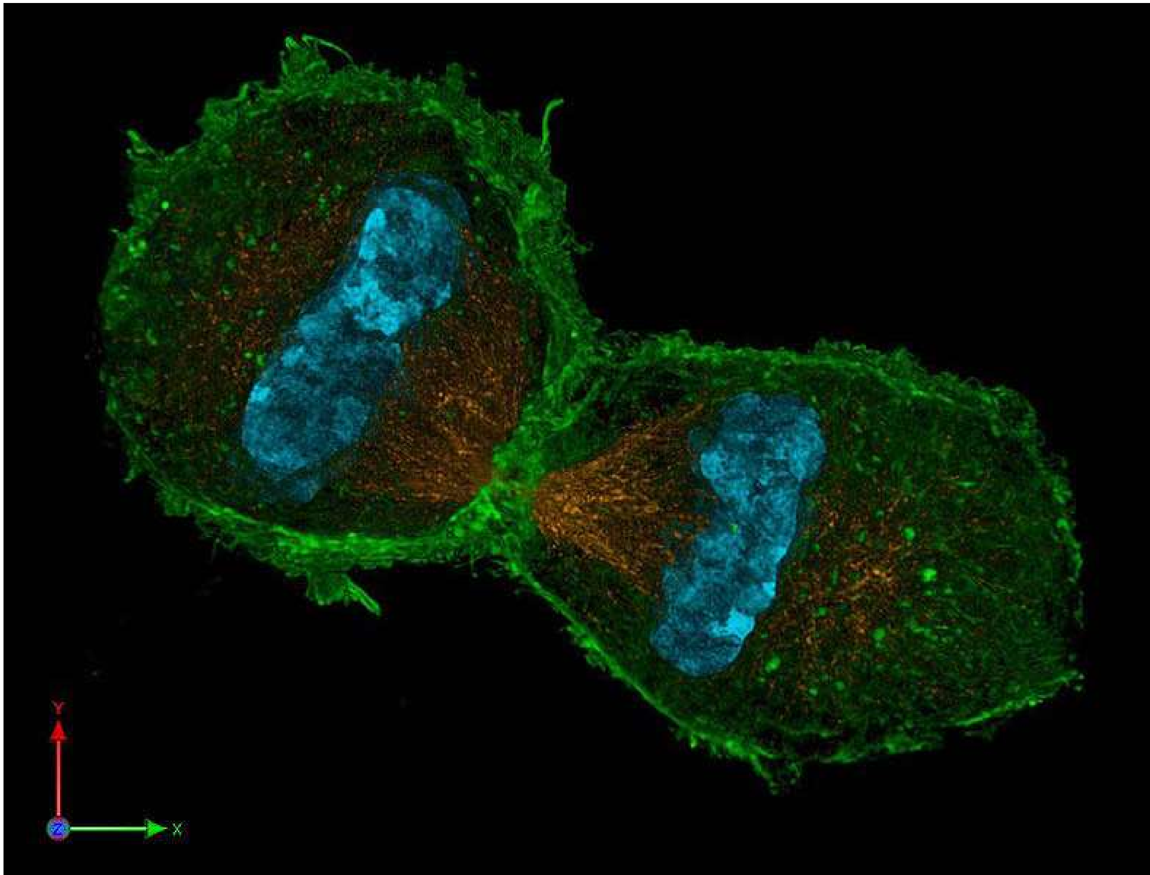
Comparison confocal microscopy - 3D-SIM



Cell nucleus in prophase from various angles



Two mouse cell nuclei in prophase.



mouse cell in telophase

Extensions

Most modern instruments provide simple solutions for micro-photography and image recording electronically. However such capabilities are not always present and the more experienced microscopist will, in many cases, still prefer a hand drawn image rather than a photograph. This is because a microscopist with knowledge of the subject can accurately convert a three dimensional image into a precise two dimensional drawing . In a photograph or other image capture system however, only one thin plane is ever in good focus.

The creation of careful and accurate micrographs requires a microscopical technique using a monocular eyepiece. It is essential that both eyes are open and that the eye that is not observing down the microscope is instead concentrated on a sheet of paper on the bench besides the microscope. With practice, and without moving the head or eyes, it is possible to accurately record the observed details by tracing round the observed shapes by simultaneously "seeing" the pencil point in the microscopical image.

Practicing this technique also establishes good general microscopical technique. It is always less tiring to observe with the microscope focused so that the image is seen at infinity and with both eyes open at all times.

X-ray

As resolution depends on the wavelength of the light. Electron microscopy has been developed since the 1930s that use electron beams instead of light. Because of the much smaller wavelength of the electron beam, resolution is far higher.

Though less common, X-ray microscopy has also been developed since the late 1940s. The resolution of X-ray microscopy lies between that of light microscopy and electron microscopy.

Electron microscopy

For light microscopy, the wavelength of the light limits the resolution to around 0.2 micrometers. In order to gain higher resolution, the use of an electron beam with a far smaller wavelength is used in electron microscopes.

- Transmission electron microscopy (TEM) is quite similar to the compound light microscope, by sending an electron beam through a very thin slice of the specimen. The resolution limit in 2005 was around 0.05 nanometer and has not increased appreciably since that time.
- Scanning electron microscopy (SEM) visualizes details on the surfaces of cells and particles and gives a very nice 3D view. It gives results much like those of the stereo light microscope, and, akin to that, its most useful magnification is in the lower range than that of the transmission electron microscope.

Atomic de Broglie

The *atomic de Broglie microscope* is an imaging system which is expected to provide resolution at the nanometer scale using neutral He atoms as probe particles. . Such a device could provide the resolution at nanometer scale and be absolutely non-destructive, but it is not developed as well as optical or electron microscopes.

Scanning probe microscopy

This is a sub-diffraction technique. Examples of scanning probe microscopes are the atomic force microscope (AFM), the Scanning tunneling microscope and the photonic force microscope. All such methods imply a solid probe tip in the vicinity (near field) of an object, which is supposed to be almost flat.

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Ultrasonic force

Ultrasonic Force Microscopy (UFM) has been developed in order to improve the details and image contrast on "flat" areas of interest where the AFM images are limited in contrast. The combination of AFM-UFM allows a near field acoustic microscopic image to be generated. The AFM tip is used to detect the ultrasonic waves and overcomes the limitation of wavelength that occurs in acoustic microscopy. By using the elastic changes under the AFM tip, an image of much greater detail than the AFM topography can be generated.

Ultrasonic force microscopy allows the local mapping of elasticity in atomic force microscopy by the application of ultrasonic vibration to the cantilever or sample. In an attempt to analyze the results of ultrasonic force microscopy in a quantitative fashion, a force-distance curve measurement is done with ultrasonic vibration applied to the cantilever base, and the results are compared with a model of the cantilever dynamics and tip-sample interaction based on the finite-difference technique.

Infrared microscopy

The term *infrared microscope* covers two main types of diffraction-limited microscopy. The first provides optical visualization plus IR spectroscopic data collection. The second (more recent and more advanced) technique employs *focal plane array detection* for infrared chemical imaging, where the image contrast is determined by the response of individual sample regions to particular IR wavelengths selected by the user.

IR versions of sub-diffraction microscopy exist also. These include IR NSOM and photothermal microspectroscopy.

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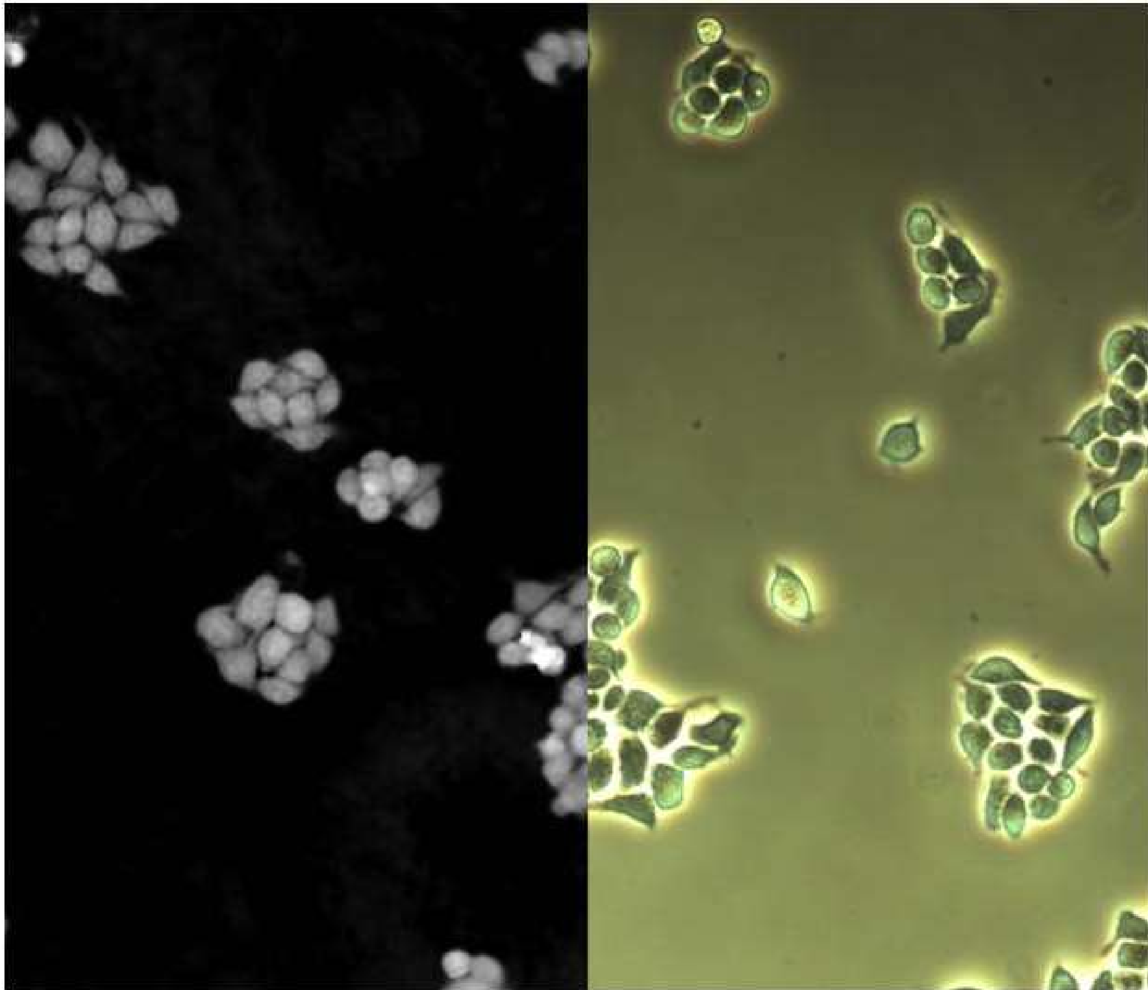
Digital holographic microscopy



Human cells imaged by DHM phase shift (left) and phase contrast microscopy (right).

In digital holographic microscopy (DHM), interfering wave fronts from a coherent (monochromatic) light-source are recorded on a sensor. The image is digitally reconstructed by a computer from the recorded hologram. Besides the ordinary bright field image, a phase shift image is created as well.

DHM can operate both in reflection and transmission mode. In reflection mode, the phase shift image provides a relative distance measurement and thus represents a topography map of the reflecting surface. In transmission mode, the phase shift image provides a label-free quantitative measurement of the optical thickness of the specimen. Phase shift images of biological cells are very similar to images of stained cells and have successfully been analyzed by high content analysis software.

A unique feature of DHM is the ability to adjust focus after the image is recorded, since all focus planes are recorded simultaneously by the hologram. This feature makes it possible to image moving particles in a volume or to rapidly scan a surface. Another attractive feature is DHM's ability to use low cost optics by correcting optical aberrations by software.

Digital Pathology (virtual microscopy)

Digital Pathology is an image-based information environment enabled by computer technology that allows for the management of information generated from a digital slide. Digital pathology is enabled in part by virtual microscopy, which is the practice of converting glass slides into digital slides that can be viewed, managed, and analyzed.

Laser microscopy

Laser microscopy is a rapidly growing field that uses laser illumination sources in various forms of microscopy. For instance, laser microscopy focused on biological applications uses ultrashort pulse lasers, or femtosecond lasers, in a number of techniques labeled as nonlinear microscopy, saturation microscopy, and multiphoton fluorescence microscopy.

Amateur microscopy

Amateur Microscopy is the investigation and observation of biological and non-biological specimens for recreational purposes. Collectors of minerals, insects, seashells, and plants may use microscopes as tools to uncover features that help them classify their collected items. Other amateurs may be interested in observing the life found in pond water and of other samples. Microscopes may also prove useful for the water quality assessment for people that keep a home aquarium. Photographic documentation and drawing of the microscopic images are additional tasks that augment the spectrum of tasks of the amateur. There are even competitions for photomicrograph art. Participants of this pastime either may use commercially prepared microscopic slides or may engage in the task of specimen preparation.

While microscopy is a central tool in the documentation of biological specimens, it is, in general, insufficient to justify the description of a new species based on microscopic investigations alone. Often genetic and biochemical tests are necessary to confirm the discovery of a new species. A laboratory and access to academic literature is a necessity, which is specialized and, in general, not available to amateurs. There is, however, one huge advantage that amateurs have above professionals: time to explore their surroundings. Often, advanced amateurs team up with professionals to validate their findings and (possibly) describe new species.

In the late 1800s, amateur microscopy became a popular hobby in the United States and Europe. Several 'professional amateurs' were being paid for their sampling trips and microscopic explorations by philanthropists, to keep them amused on the Sunday

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afternoon (e.g., the diatom specialist A. Grunow, being paid by (among others) a Belgian industrialist). Professor John Phin published "Practical Hints on the Selection and Use of the Microscope (Second Edition, 1878)," and was also the editor of the “American Journal of Microscopy.”

In 1995, a loose group of amateur microscopists, drawn from several organizations in the UK and USA, founded a site for microscopy based on the knowledge and input of amateur (perhaps better referred to as 'enthusiast') microscopists. This was the first attempt to establish 'amateur' microscopy as a serious subjectin the then-emerging new media of the Internet. Today, it remains as a powerful established international resource for all ages, to input their findings and share information.

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Chapter 14

Electron Microscope

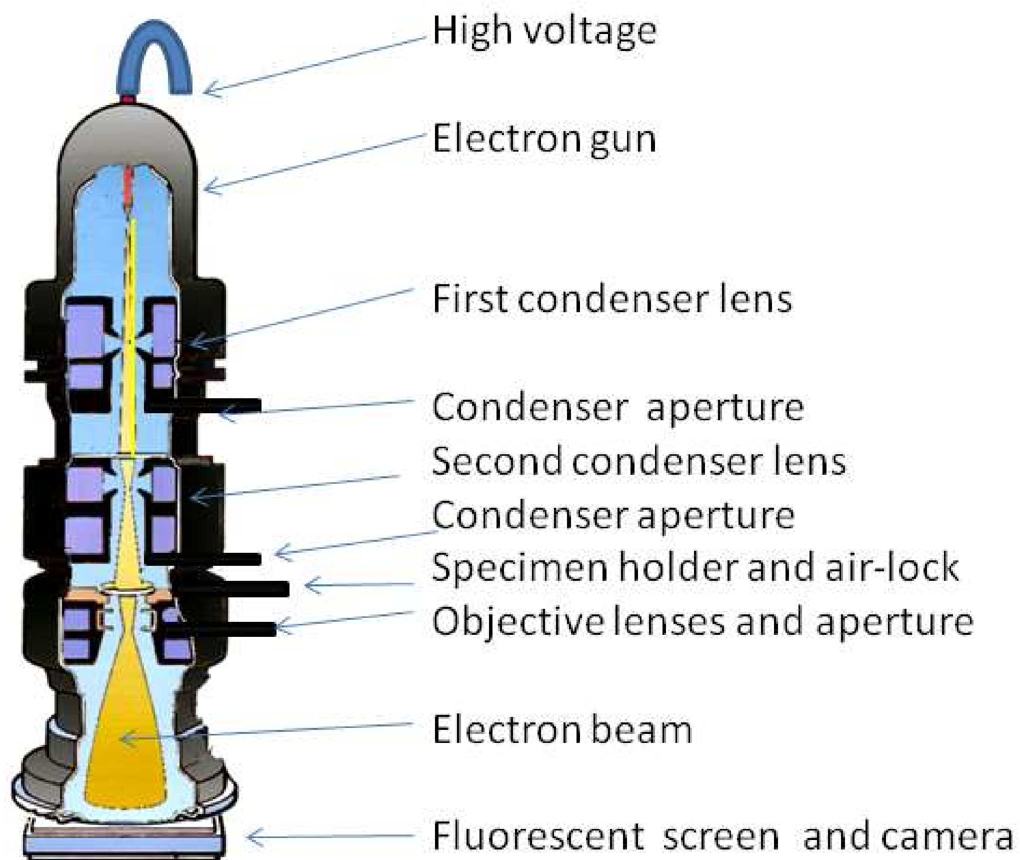


Diagram of a transmission electron microscope

A 1973 Siemens electron microscope, Musée des Arts et Métiers, Paris

An **electron microscope** is a type of microscope that uses a particle beam of electrons to illuminate the specimen and produce a magnified image. Electron microscopes (EM) have a greater resolving power than a light-powered optical microscope, because electrons have wavelengths about 100,000 times shorter than visible light (photons), and can achieve better than 0.2 nm resolution and magnifications of up to 2,000,000x, whereas ordinary, non-confocal light microscopes are limited by diffraction to about 200 nm resolution and useful magnifications below 2000x.

The electron microscope uses electrostatic and electromagnetic "lenses" to control the electron beam and focus it to form an image. These lenses are analogous to, but different from the glass lenses of an optical microscope that form a magnified image by focusing light on or through the specimen. In transmission, the electron beam is first diffracted by the specimen, and then, the electron microscope “lenses" re-focus the beam into a Fourier-transformed image of the diffraction pattern for the selected area of investigation. The real image thus formed is magnified by a factor ranging from a few hundred to many hundred thousand times, and can be viewed on a detecting screen or recorded using photographic film or plates or with a digital camera. Electron microscopes are used to

observe a wide range of biological and inorganic specimens including microorganisms, cells, large molecules, biopsy samples, metals, and crystals. Industrially, the electron microscope is primarily used for quality control and failure analysis in semiconductor device fabrication.

The advantages of electron microscopy over X-ray crystallography are that the specimen need not be a single crystal or even a polycrystalline powder, and also that the Fourier transform reconstruction of the object's magnified structure occurs physically and thus avoids the need for solving the phase problem faced by the X-ray crystallographers after obtaining their X-ray diffraction patterns of a single crystal or polycrystalline powder. The transmission electron microscope's major `disadvantage' is the need for extremely thin sections of the specimens, typically less than 100 nanometers. Biological specimens typically require to be chemically fixed, dehydrated and embedded in a polymer resin to stabilize them sufficiently to allow ultrathin sectioning. Sections of biological specimens, organic polymers and similar materials may require special `staining' with heavy atom labels in order to achieve the required image contrast.

History

Electron microscope constructed by Ernst Ruska in 1933

In 1931, the German physicist Ernst Ruska and German electrical engineer Max Knoll constructed the prototype electron microscope, capable of four-hundred-power magnification; the apparatus was a practical application of the principles of electron microscopy. Two years later, in 1933, Ruska built an electron microscope that exceeded the resolution attainable with an optical (lens) microscope. Moreover, Reinhold Rudenberg, the scientific director of Siemens-Schuckertwerke, obtained the patent for the electron microscope in May 1931. Family illness compelled the electrical engineer to devise an electrostatic microscope, because he wanted to make visible the poliomyelitis virus.

In 1937, the Siemens company financed the development work of Ernst Ruska and Bodo von Borries, and employed Helmut Ruska (Ernst's brother) to develop applications for the microscope, especially with biologic specimens. Also in 1937, Manfred von Ardenne pioneered the scanning electron microscope. The first *practical* electron microscope was constructed in 1938, at the University of Toronto, by Eli Franklin Burton and students Cecil Hall, James Hillier, and Albert Prebus; and Siemens produced the first *commercial* Transmission Electron Microscope (TEM) in 1939. Although contemporary electron microscopes are capable of two million-power magnification, as scientific instruments, they remain based upon Ruska's prototype.

Types

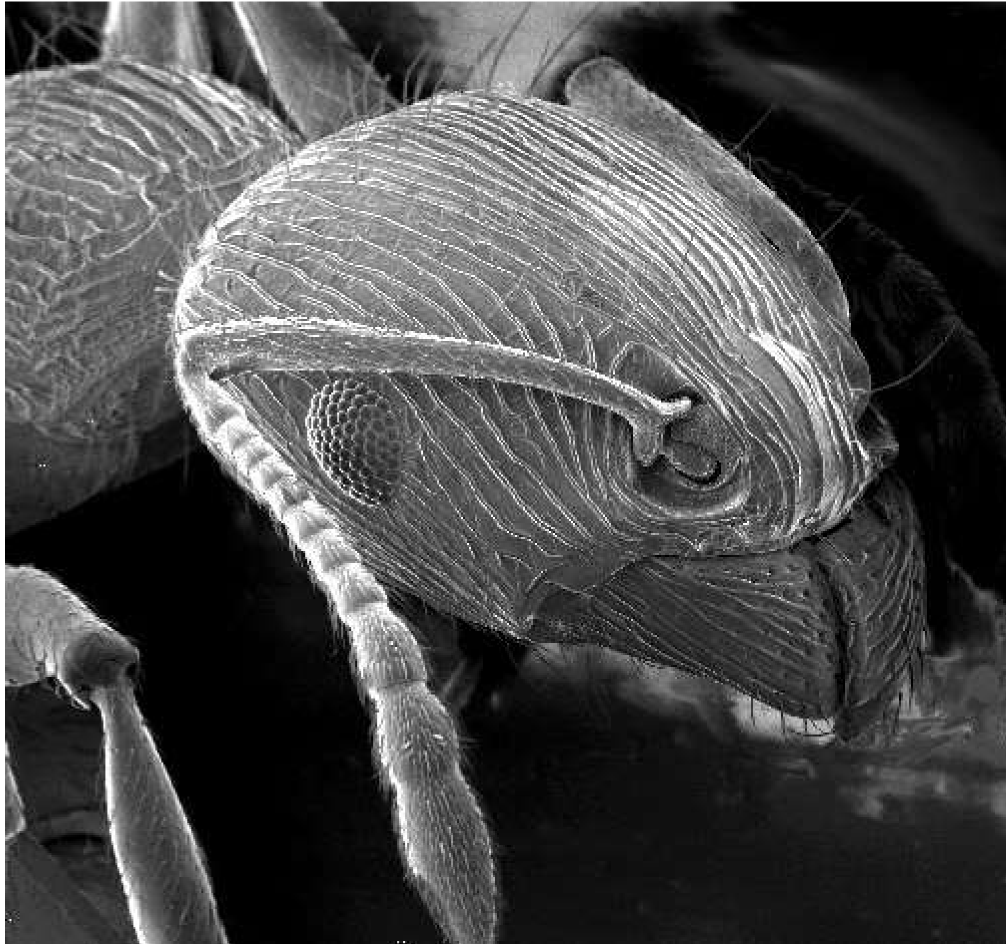
Transmission electron microscope (TEM)

The original form of electron microscope, the transmission electron microscope (TEM) uses a high voltage electron beam to create an image. The electrons are emitted by an electron gun, commonly fitted with a tungsten filament cathode as the electron source. The electron beam is accelerated by an anode typically at +100 keV (40 to 400 keV) with respect to the cathode, focused by electrostatic and electromagnetic lenses, and transmitted through the specimen that is in part transparent to electrons and in part scatters them out of the beam. When it emerges from the specimen, the electron beam carries information about the structure of the specimen that is magnified by the objective lens system of the microscope. The spatial variation in this information (the "image") is viewed by projecting the magnified electron image onto a fluorescent viewing screen coated with a phosphor or scintillator material such as zinc sulfide. The image can be photographically recorded by exposing a photographic film or plate directly to the electron beam, or a high-resolution phosphor may be coupled by means of a lens optical system or a fibre optic light-guide to the sensor of a CCD (charge-coupled device) camera. The image detected by the CCD may be displayed on a monitor or computer.

Resolution of the TEM is limited primarily by spherical aberration, but a new generation of aberration correctors have been able to partially overcome spherical aberration to increase resolution. Hardware correction of spherical aberration for the High Resolution TEM (HRTEM) has allowed the production of images with resolution below 0.5 Ångström (50 picometres) at magnifications above 50 million times. The ability to

determine the positions of atoms within materials has made the HRTEM an important tool for nano-technologies research and development.

Scanning electron microscope (SEM)



An image of an ant in a scanning electron microscope

Unlike the TEM, where electrons of the high voltage beam carry the image of the specimen, the electron beam of the Scanning Electron Microscope (SEM) does not at any time carry a complete image of the specimen. The SEM produces images by probing the specimen with a focused electron beam that is scanned across a rectangular area of the specimen (raster scanning). At each point on the specimen the incident electron beam loses some energy, and that lost energy is converted into other forms, such as heat, emission of low-energy secondary electrons, light emission (cathodoluminescence) or x-ray emission. The display of the SEM maps the varying intensity of any of these signals into the image in a position corresponding to the position of the beam on the specimen when the signal was generated. In the SEM image of an ant shown at right, the image was constructed from signals produced by a secondary electron detector, the normal or conventional imaging mode in most SEMs.

Generally, the image resolution of an SEM is about an order of magnitude poorer than that of a TEM. However, because the SEM image relies on surface processes rather than transmission, it is able to image bulk samples up to many centimetres in size and (depending on instrument design and settings) has a great depth of field, and so can produce images that are good representations of the three-dimensional shape of the sample.

Reflection electron microscope (REM)

In the **Reflection Electron Microscope** (REM) as in the TEM, an electron beam is incident on a surface, but instead of using the transmission (TEM) or secondary electrons (SEM), the reflected beam of elastically scattered electrons is detected. This technique is typically coupled with Reflection High Energy Electron Diffraction (RHEED) and *Reflection high-energy loss spectrum (RHELS)*. Another variation is Spin-Polarized Low-Energy Electron Microscopy (SPLEEM), which is used for looking at the microstructure of magnetic domains.

Scanning transmission electron microscope (STEM)

The STEM rasters a focused incident probe across a specimen that (as with the TEM) has been thinned to facilitate detection of electrons scattered *through* the specimen. The high resolution of the TEM is thus possible in STEM. The focusing action (and aberrations) occur before the electrons hit the specimen in the STEM, but afterward in the TEM. The STEMs use of SEM-like beam rastering simplifies annular dark-field imaging, and other analytical techniques, but also means that image data is acquired in serial rather than in parallel fashion.

Low-voltage electron microscope (LVEM)

The low-voltage electron microscope (LVEM) is a combination of SEM, TEM and STEM in one instrument, which operates at relatively low electron accelerating voltage of 5 kV. Low voltage increases image contrast which is especially important for biological specimens. This increase in contrast significantly reduces, or even eliminates the need to stain. Sectioned samples generally need to be thinner than they would be for conventional TEM (20-65 nm). Resolutions of a few nm are possible in TEM, SEM and STEM modes.

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Sample preparation

An insect coated in gold for viewing with a scanning electron microscope.

Materials to be viewed under an electron microscope may require processing to produce a suitable sample. The technique required varies depending on the specimen and the analysis required:

- *Chemical fixation* - for biological specimens aims to stabilize the specimen's mobile macromolecular structure by chemical crosslinking of proteins with aldehydes such as formaldehyde and glutaraldehyde, and lipids with osmium tetroxide.

- *Negative stain* - suspensions containing fine biological material (such as viruses and bacteria) are briefly mixed with a dilute solution of an electron-opaque solution such as ammonium molybdate, uranyl acetate (or formate), or phosphotungstic acid. This mixture is applied to a suitably-coated EM grid, blotted, then allowed to dry. Viewing of this preparation in the TEM should be carried out without delay for best results. The method is important in microbiology for fast but crude morphological identification, but can also be used as the basis for high resolution 3D reconstruction using EM tomography methodology when carbon films are used for support.
- *Cryofixation* – freezing a specimen so rapidly, to liquid nitrogen or even liquid helium temperatures, that the water forms vitreous (non-crystalline) ice. This preserves the specimen in a snapshot of its solution state. An entire field called cryo-electron microscopy has branched from this technique. With the development of cryo-electron microscopy of vitreous sections (CEMOVIS), it is now possible to observe samples from virtually any biological specimen close to its native state.
- *Dehydration* – freeze drying, or replacement of water with organic solvents such as ethanol or acetone, followed by critical point drying or infiltration with embedding resins.
- *Embedding, biological specimens* – after dehydration, tissue for observation in the transmission electron microscope is embedded so it can be sectioned ready for viewing. To do this the tissue is passed through a 'transition solvent' such as epoxy propane and then infiltrated with a resin such as Araldite epoxy resin; tissues may also be embedded directly in water-miscible acrylic resin. After the resin has been polymerised (hardened) the sample is thin sectioned (ultrathin sections) and stained - it is then ready for viewing.
- *Embedding, materials* - after embedding in resin, the specimen is usually ground and polished to a mirror-like finish using ultra-fine abrasives. The polishing process must be performed carefully to minimize scratches and other polishing artifacts that reduce image quality.
- *Sectioning* – produces thin slices of specimen, semitransparent to electrons. These can be cut on an ultramicrotome with a diamond knife to produce ultrathin slices about 60-90 nm thick. Disposable glass knives are also used because they can be made in the lab and are much cheaper.
- *Staining* – uses heavy metals such as lead, uranium or tungsten to scatter imaging electrons and thus give contrast between different structures, since many (especially biological) materials are nearly "transparent" to electrons (weak phase objects). In biology, specimens are can be stained "en bloc" before embedding and also later after sectioning. Typically thin sections are stained for several minutes with an aqueous or alcoholic solution of uranyl acetate followed by aqueous lead citrate.
- *Freeze-fracture or freeze-etch* – a preparation method particularly useful for examining lipid membranes and their incorporated proteins in "face on" view. The fresh tissue or cell suspension is frozen rapidly (cryofixation), then fractured by simply breaking or by using a microtome while maintained at liquid nitrogen temperature. The cold fractured surface (sometimes "etched" by increasing the

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temperature to about –100 °C for several minutes to let some ice sublime) is then shadowed with evaporated platinum or gold at an average angle of 45° in a high vacuum evaporator. A second coat of carbon, evaporated perpendicular to the average surface plane is often performed to improve stability of the replica coating. The specimen is returned to room temperature and pressure, then the extremely fragile "pre-shadowed" metal replica of the fracture surface is released from the underlying biological material by careful chemical digestion with acids, hypochlorite solution or SDS detergent. The still-floating replica is thoroughly washed free from residual chemicals, carefully fished up on fine grids, dried then viewed in the TEM.

- *Ion Beam Milling* – thins samples until they are transparent to electrons by firing ions (typically argon) at the surface from an angle and sputtering material from the surface. A subclass of this is Focused ion beam milling, where gallium ions are used to produce an electron transparent membrane in a specific region of the sample, for example through a device within a microprocessor. Ion beam milling may also be used for cross-section polishing prior to SEM analysis of materials that are difficult to prepare using mechanical polishing.
- *Conductive Coating* – an ultrathin coating of electrically-conducting material, deposited either by high vacuum evaporation or by low vacuum sputter coating of the sample. This is done to prevent the accumulation of static electric fields at the specimen due to the electron irradiation required during imaging. Such coatings include gold, gold/palladium, platinum, tungsten, graphite etc. and are especially important for the study of specimens with the scanning electron microscope. Another reason for coating, even when there is more than enough conductivity, is to improve contrast, a situation more common with the operation of a FESEM (field emission SEM).

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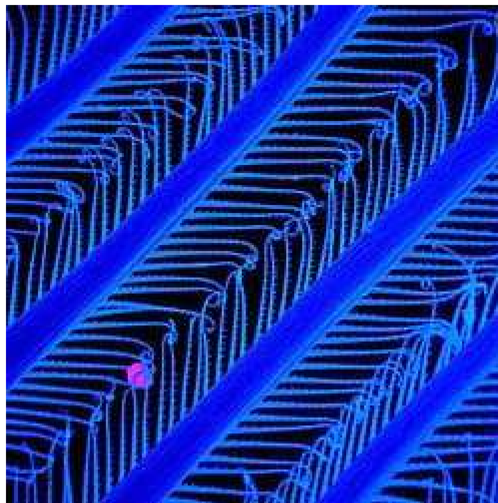
Disadvantages



False-color SEM image of the filter setae of an Antarctic krill. (Raw electron microscope images carry no color information.)

Pictured: First degree filter setae with V-shaped second degree setae pointing towards the inside of the feeding basket. The purple ball is 1 μm in diameter.

Electron microscopes are expensive to build and maintain, but the capital and running costs of confocal light microscope systems now overlaps with those of basic electron microscopes. They are dynamic rather than static in their operation, requiring extremely stable high-voltage supplies, extremely stable currents to each electromagnetic coil/lens, continuously-pumped high- or ultra-high-vacuum systems, and a cooling water supply circulation through the lenses and pumps. As they are very sensitive to vibration and external magnetic fields, microscopes designed to achieve high resolutions must be housed in stable buildings (sometimes underground) with special services such as magnetic field cancelling systems. Some desktop low-voltage electron microscopes have TEM capabilities at very low voltages (around 5 kV) without stringent voltage supply, lens coil current, cooling water or vibration isolation requirements and as such are much less expensive to buy and far easier to install and maintain, but do not have the same ultra-high (atomic scale) resolution capabilities as the larger instruments.

The samples largely have to be viewed in vacuum, as the molecules that make up air would scatter the electrons. One exception is the environmental scanning electron microscope, which allows hydrated samples to be viewed in a low-pressure (up to 20 Torr/2.7 kPa), wet environment.

Scanning electron microscopes usually image conductive or semi-conductive materials best. Non-conductive materials can be imaged by an environmental scanning electron microscope. A common preparation technique is to coat the sample with a several-nanometer layer of conductive material, such as gold, from a sputtering machine; however, this process has the potential to disturb delicate samples.

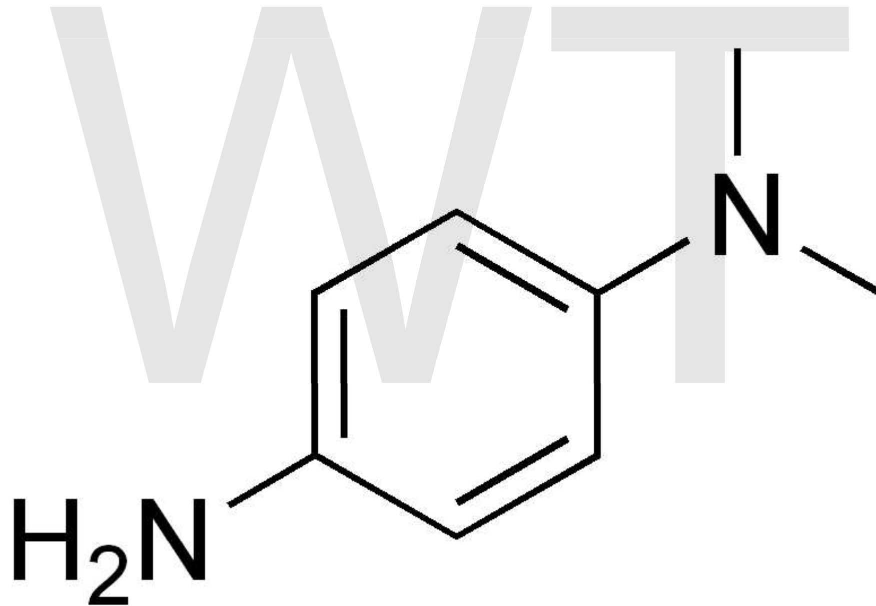
Small, stable specimens such as carbon nanotubes, diatom frustules and small mineral crystals (asbestos fibres, for example) require no special treatment before being examined in the electron microscope. Samples of hydrated materials, including almost all biological specimens have to be prepared in various ways to stabilize them, reduce their thickness (ultrathin sectioning) and increase their electron optical contrast (staining). These processes may result in *artifacts*, but these can usually be identified by comparing the results obtained by using radically different specimen preparation methods. It is generally believed by scientists working in the field that as results from various preparation techniques have been compared and that there is no reason that they should all produce similar artifacts, it is reasonable to believe that electron microscopy features correspond with those of living cells. In addition, higher-resolution work has been directly compared to results from X-ray crystallography, providing independent confirmation of the validity of this technique. Since the 1980s, analysis of cryofixed, vitrified specimens has also become increasingly used by scientists, further confirming the validity of this technique.

WORLD TECHNOLOGIES

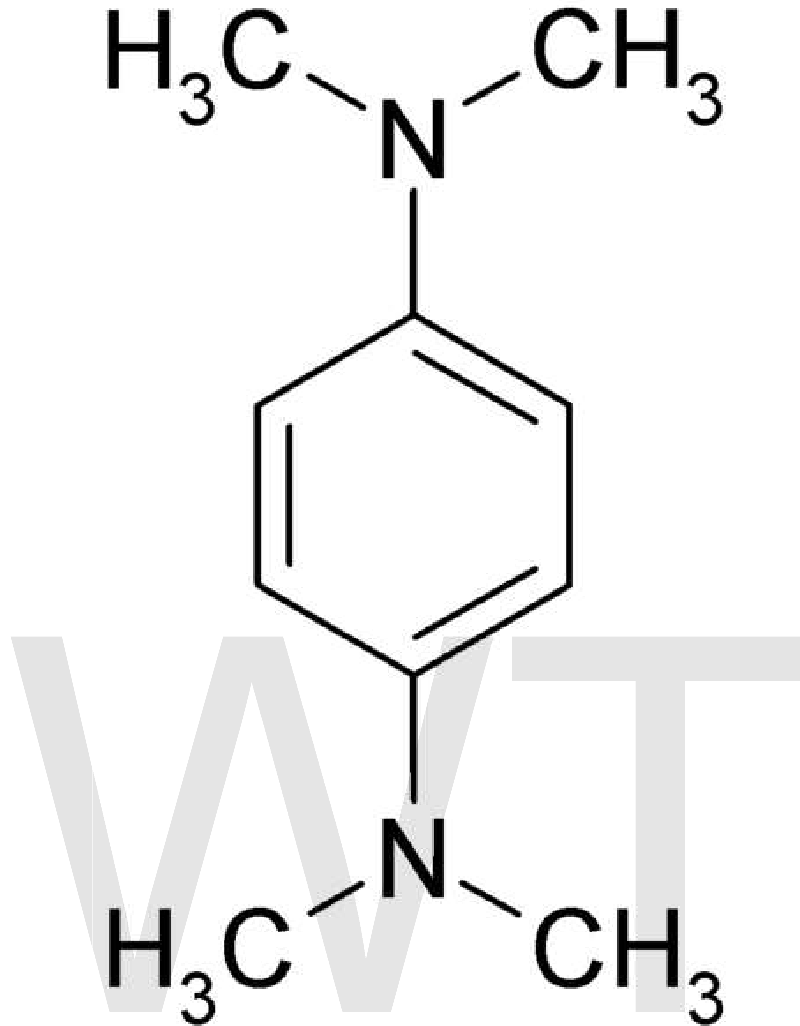
Chapter 15

Oxidase Test, Isopycnic Centrifugation and Microbiological Culture

Oxidase test



DMPD



TMPD

The **oxidase test** is a test used in microbiology to determine if a bacterium produces certain cytochrome c oxidases. It uses disks impregnated with a reagent such as *N,N,N′,N′*-tetramethyl-*p*-phenylenediamine (TMPD) or *N,N*-Dimethyl-*p*-phenylenediamine (DMPD), which is also a redox indicator. The reagent is a dark blue to maroon color when oxidized, and colorless when reduced.

Classification

Strains may either be oxidase positive (OX+) or negative (OX-).

OX+

OX+ normally means that the bacterium contains cytochrome c oxidase and can therefore utilize oxygen for energy production with an electron transfer chain.

Typically the Pseudomonadaceae are OX+

Another example is the preliminary identification of *Neisseria* and *Moraxella* genera, which are both oxidase positive, Gram-negative diplococci.

Many Gram-negative spiral curved rods are also oxidase positive, which includes *Helicobacter pylori*, *Vibrio cholera*, and *Campylobacter jejuni*.

Also *Legionella pneumophila* is oxidase positive. A trick to remember the most medical relevant bacteria is: "VIce President CHeNEy MOstly LEads" (*Vibrio*, *Pseudomonas*, *Campylobacter*, *Helicobacter*, *Neisseria*, *Moraxella*, and *Legionella*, respectively).

OX-

OX- normally means that the bacterium does not contain cytochrome c oxidase and therefore cannot utilize oxygen for energy production with an electron transfer chain.

Typically Enterobacteriaceae are OX-.

Procedures

1. Wet each disk with about 4 inoculating loops of de-ionized water.
2. Use a loop to aseptically transfer a large mass of pure bacteria to the disk.
3. Observe the disk for up to 3 minutes. If the area of inoculation turns dark blue to maroon to almost black, then the result is positive. If a color change does not occur within three minutes, the result is negative.

Alternatively, live bacteria cultivated on trypticase soy agar plates may be prepared using sterile technique with a single-line streak inoculation. The inoculated plates are incubated at 37°C for 24–48 hours to establish colonies. Fresh bacterial preparations should be used. After colonies have grown on the media, two-to-three drops of the reagent DMPD is added to the surface of each organism to be tested.

- A positive test (OX+) will result in a color change to pink, through maroon and into black, within 10–30 seconds.
- A negative test (OX-) will result in a light pink coloration or absence of coloration.

Isopycnic centrifugation

Isopycnic centrifugation is a technique used to separate molecules on the basis of density. (The word "isopycnic" means "equal density".) Typically, a "self-generating"

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density gradient is established via equilibrium sedimentation, and then analyte molecules concentrate as bands where the molecule density matches that of the surrounding solution. To illustrate the process, consider the fractionation of nucleic acids such as DNA. To begin the analysis, a mixture of caesium chloride and DNA is placed in a centrifuge for several hours at high speed to generate a force of about 10^5 x g (earth's gravity). Caesium chloride is used because at a concentration of 1.6 to 1.8 g/mL it is similar to the density of DNA. After some time a gradient of the caesium ions is formed, caused by two opposing forces: diffusion and centrifugal force. The sedimenting particles (caesium ions) will sediment away from the rotor, and become more concentrated near the bottom of the tube. The diffusive force arises due to the concentration gradient of solvated caesium chloride and is always directed towards the center of the rotor. The balance between these two forces generates a stable density gradient in the caesium chloride solution, which is more dense near the bottom of the tube, and less dense near the top.

The DNA molecules will then be separated based on the relative proportions of AT (adenine and thymine base pairs) to GC (guanine and cytosine base pairs). An AT base pair has a lower molecular weight than a GC base pair and therefore, for two DNA molecules of equal length, the one with the greater proportion of AT base pairs will have a lower density, all other factors being equal. Different types of nucleic acids will also be separated into bands, e.g. RNA is denser than supercoiled plasmid DNA, which is denser than linear chromosomal DNA.

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Microbiological culture

A culture of *Bacillus anthracis*

A **microbiological culture**, or **microbial culture**, is a method of multiplying microbial organisms by letting them reproduce in predetermined culture media under controlled laboratory conditions. Microbial cultures are used to determine the type of organism, its abundance in the sample being tested, or both. It is one of the primary diagnostic methods of microbiology and used as a tool to determine the cause of infectious disease by letting the agent multiply in a predetermined medium. For example, a throat culture is taken by scraping the lining of tissue in the back of the throat and blotting the sample into a medium to be able to screen for harmful microorganisms, such as *Streptococcus pyogenes*, the causative agent of strep throat. Furthermore, the term culture is more generally used informally to refer to "selectively growing" a specific kind of microorganism in the lab.

Microbial cultures are foundational and basic diagnostic methods used extensively as a research tool in molecular biology. It is often essential to isolate a pure culture of microorganisms. A pure (or *axenic*) culture is a population of cells or multicellular organisms growing in the absence of other species or types. A pure culture may originate from a single cell or single organism, in which case the cells are genetic clones of one another.

For the purpose of gelling the microbial culture, the medium of agarose gel (agar) is used. Agar is a gelatinous substance derived from seaweed. A cheap substitute for agar is guar gum, which can be used for the isolation and maintenance of thermophiles.

Bacterial culture

Microbiological cultures use petri dishes of differing sizes that have a thin layer of agar-based growth medium in them. Once the growth medium in the petri dish is inoculated with the desired bacteria, the plates are incubated in an incubator (usually set at 37 degrees Celsius for cultures from humans or animals, or lower for environmental cultures). Another method of bacterial culture is liquid culture, in which the desired bacteria are suspended in liquid broth, a nutrient medium. These are ideal for preparation of an antimicrobial assay. The experimenter would inoculate liquid broth with bacteria and let it grow overnight in a shaker for uniform growth, then take aliquots of the sample to test for the antimicrobial activity of a specific drug or protein (antimicrobial peptides).

Virus and phage culture

Virus or phage cultures require host cells in which the virus or phage multiply. For bacteriophages, cultures are grown by infecting bacterial cells. The phage can then be isolated from the resulting plaques in a lawn of bacteria on a plate. Virus cultures are obtained from their appropriate eukaryotic host cells.

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Eukaryotic cell culture

Isolation of pure cultures

For single-celled eukaryotes, such as yeast, the isolation of pure cultures uses the same techniques as for bacterial cultures. Pure cultures of multicellular organisms are often more easily isolated by simply picking out a single individual to initiate a culture. This is a useful technique for pure culture of fungi, multicellular algae, and small metazoa, for example.

Developing pure culture techniques is crucial to the observation of the specimen in question. The most common method to isolate individual cells and produce a pure culture is to prepare a streak plate. The streak plate method is a way to physically separate the microbial population, and is done by spreading the inoculate back and forth with an inoculating loop over the solid agar plate. Upon incubation, colonies will arise and, hopefully, single cells will have been isolated from the biomass.

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