Microbiology Techniques

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Digital Holographic Microscopy

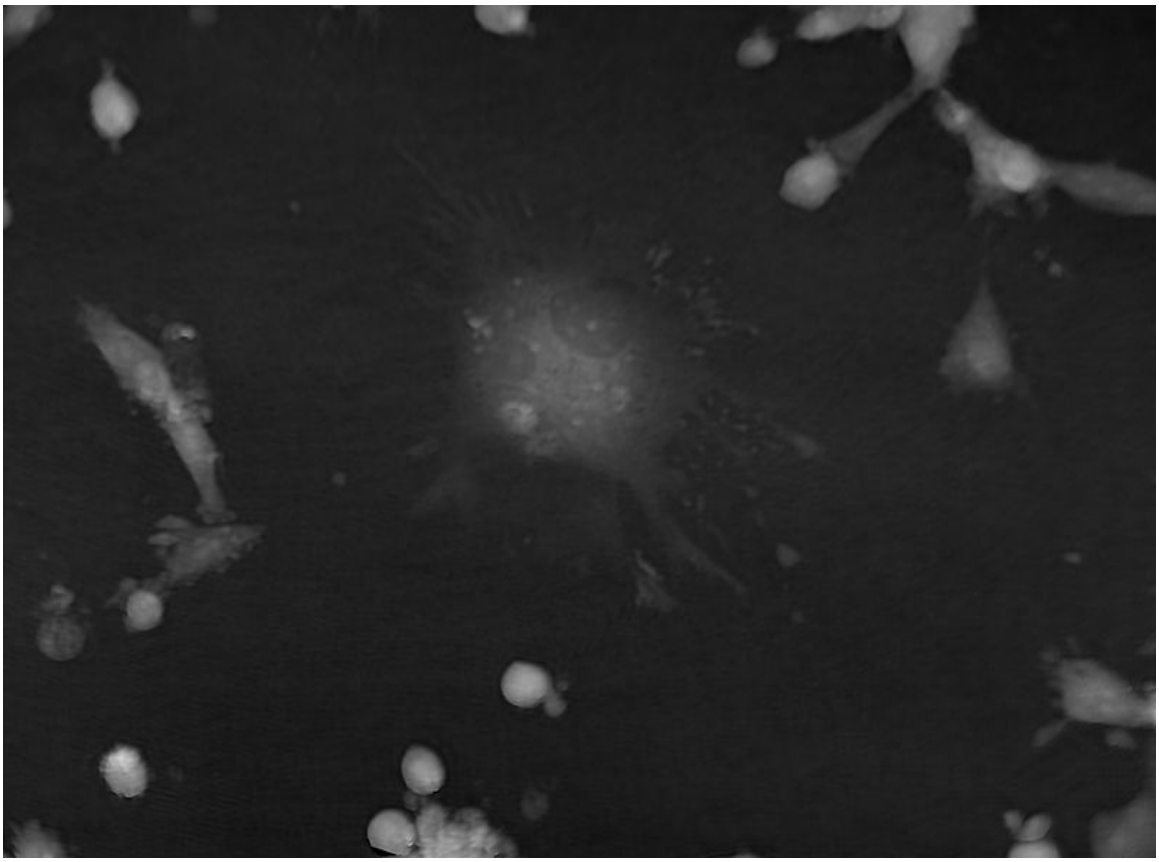


Figure 1. DHM phase shift image of cell details.

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

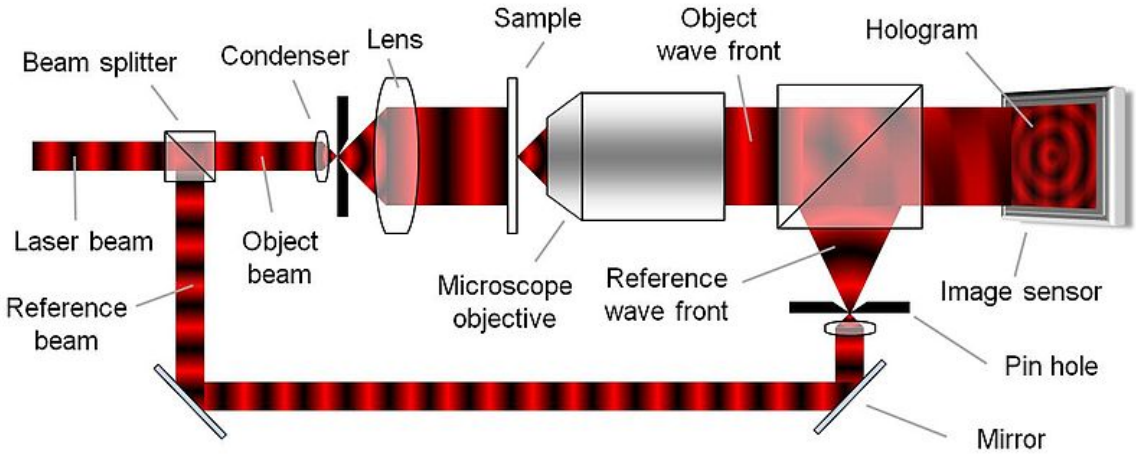


Figure 2. Typical optical setup of DHM.

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

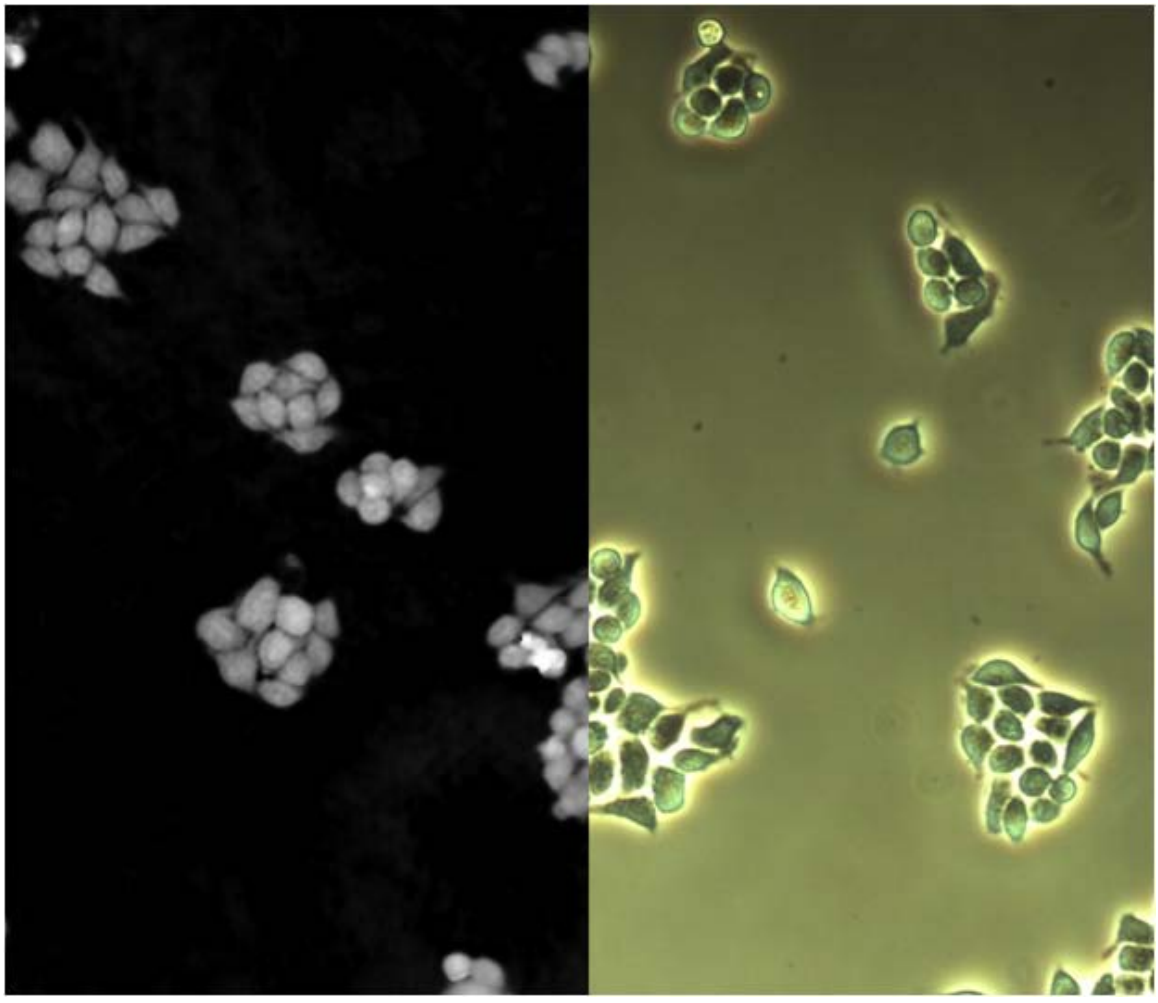


Figure 3. Comparison of a DHM phase shift image (left) and a phase contrast microscopy image (right).

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.

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

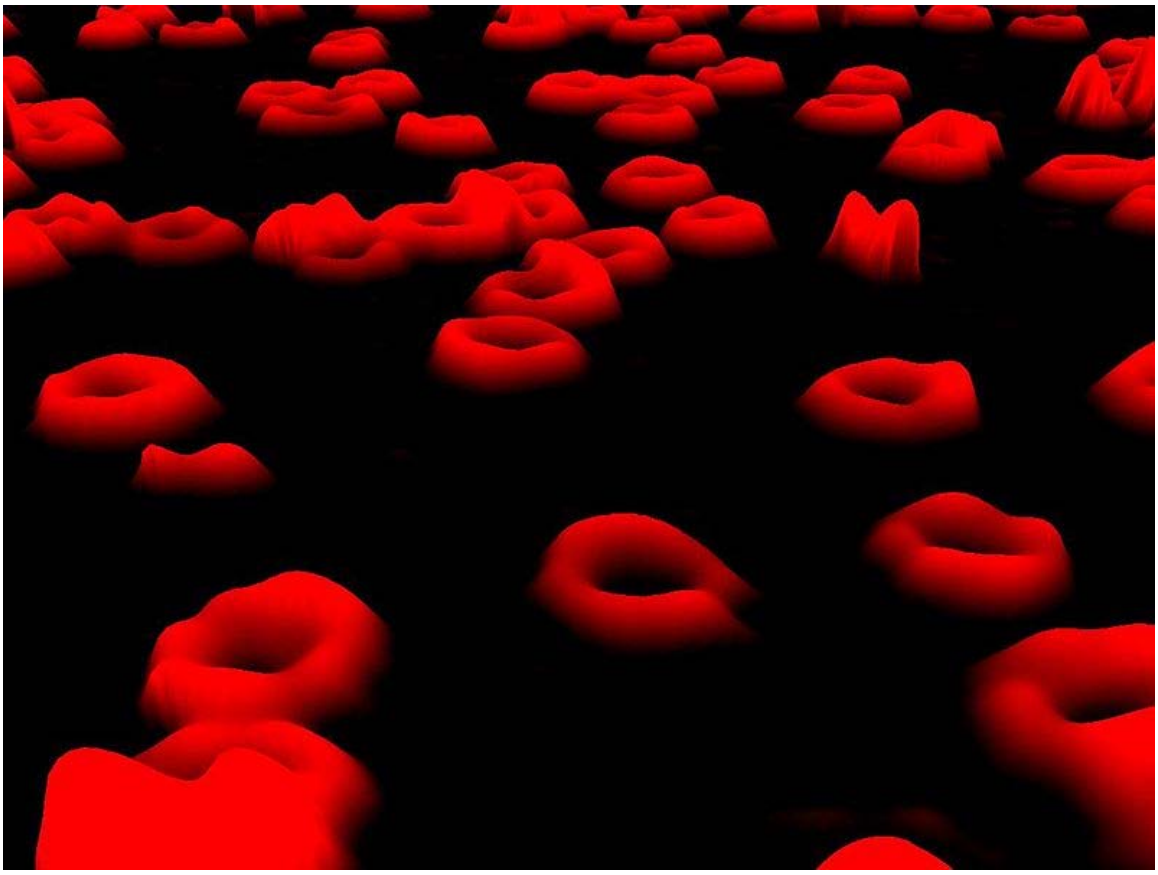


Figure 4. DHM phase shift image of human red blood cells.

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 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.

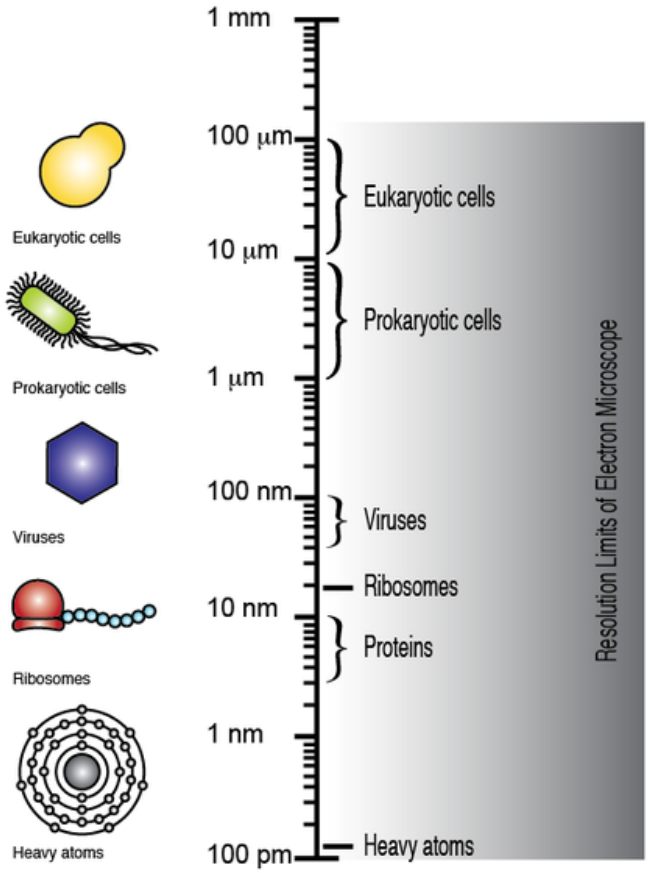
In the mid 1990s, digital image sensors and computers had become powerful enough to reconstruct images with some quality. In the 1960s, digital holography could either mean to compute an image from a hologram or to compute a hologram from a 3D model. The latter developed in parallel with classical holography 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.

Chapter- 2

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:

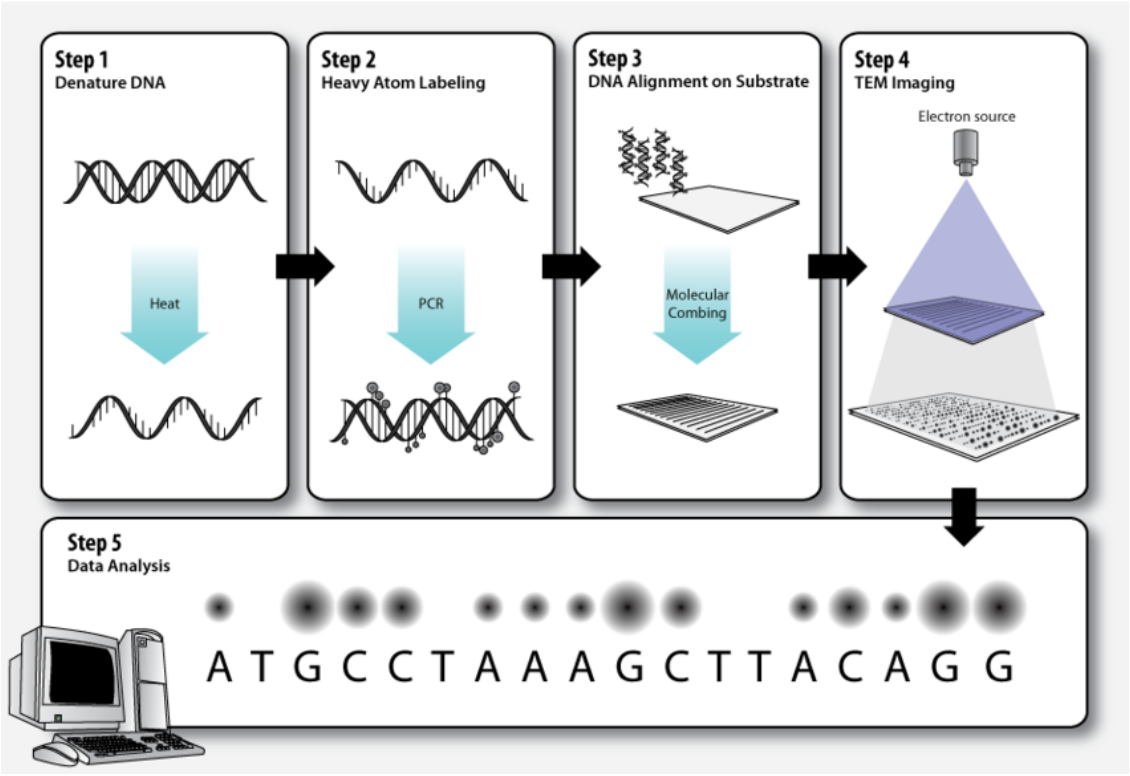
- **1970** Albert Crewe developed the high-angle annular dark-field imaging (HAADF) imaging technique in a scanning transmission electron microscope. Using this technique, he visualized individual heavy atoms on thin amorphous carbon films.
- **April 2008**: ZS Genetics presented its plans for development 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

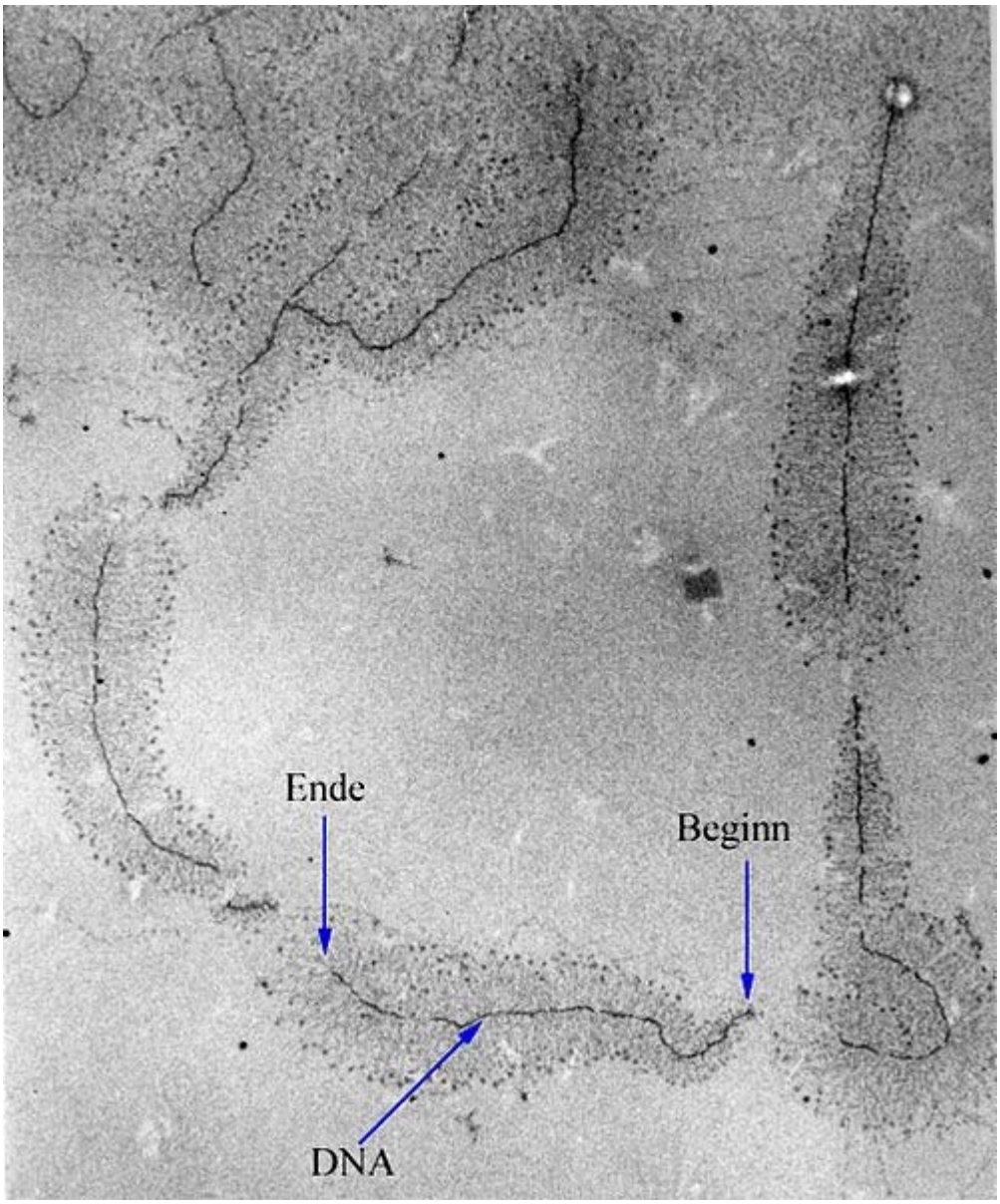
The elements that make up biological molecules (C, H, N, O, P, S) are too light (low atomic number, Z) to be clearly visualized as individual atoms by transmission electron microscopy. To circumvent this problem, the DNA bases can be labeled with heavier atoms (higher Z). Each nucleotide is tagged with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

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.

- **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**

Chapter- 3

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

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

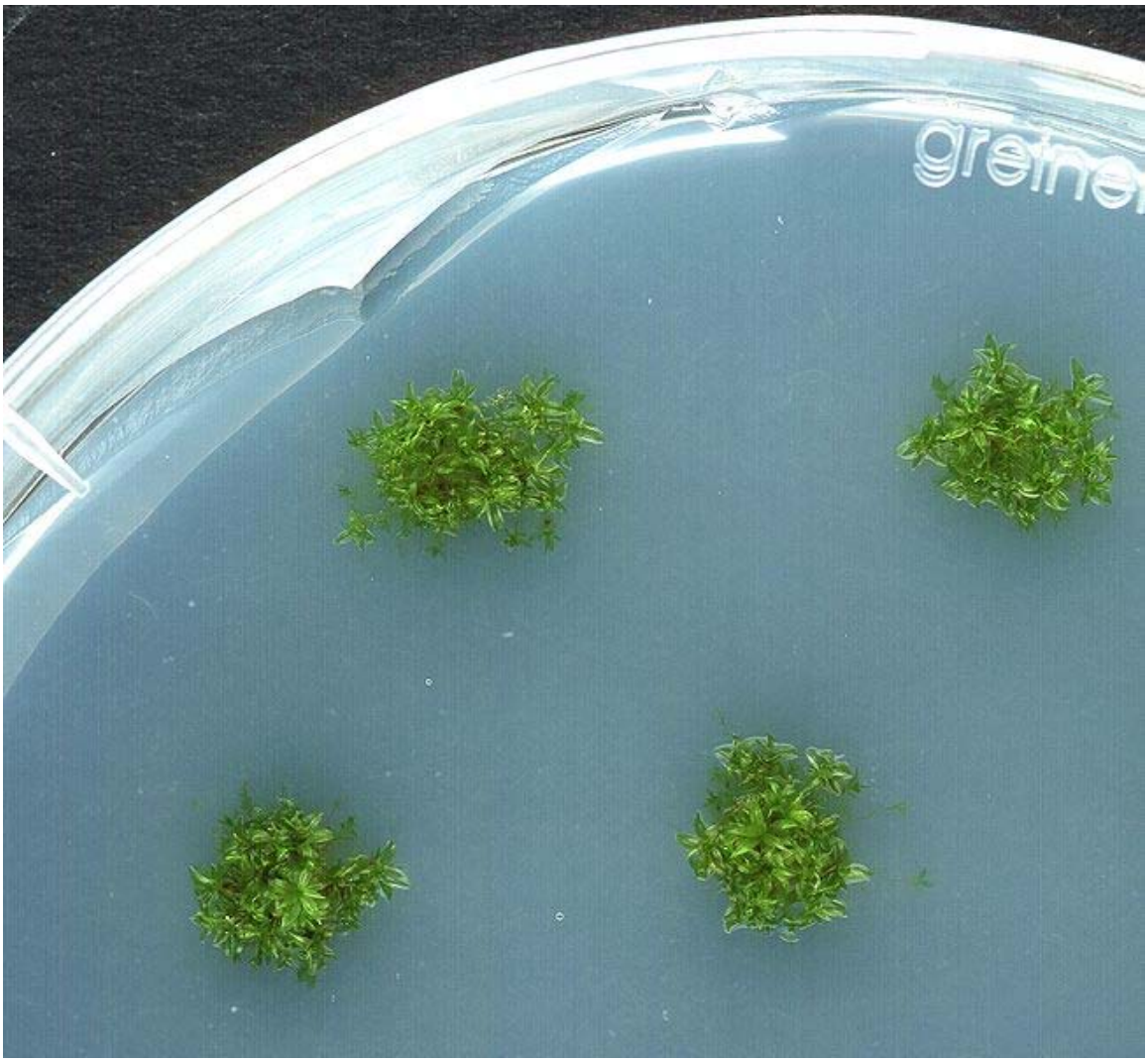
Aseptic technique

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

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- 4

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 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 *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).

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- 5

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.

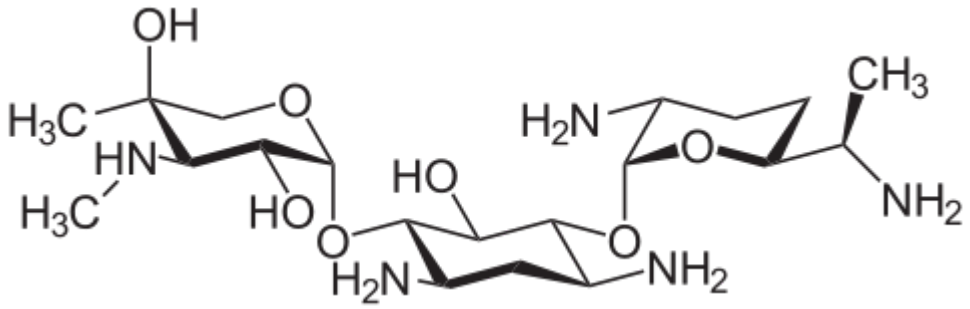
The assay is based on several observations made in the 1970s, in which the ability of internalized bacteria to avoid killing by antibiotics was reported. The assay started to be used in biological research in the early 1980s.

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.

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.

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

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 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

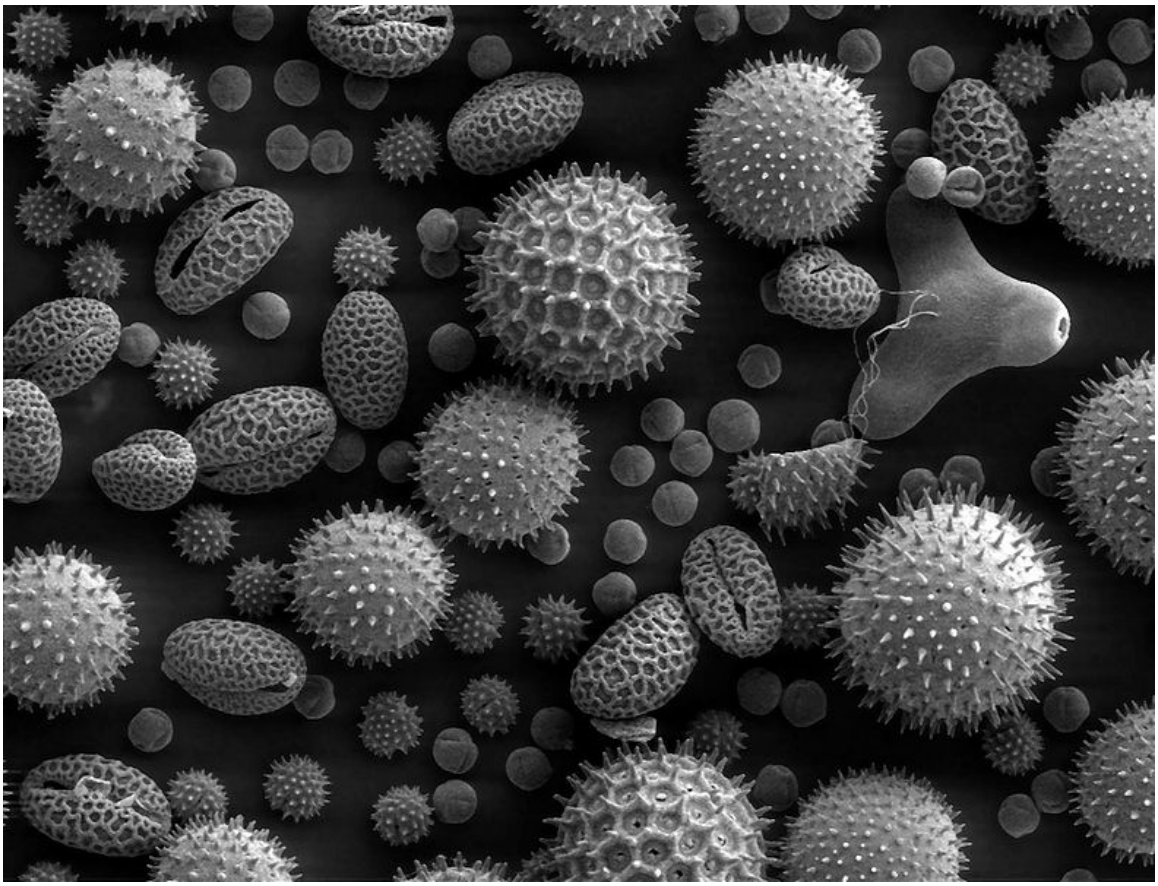
When a particular organism is introduced into a selected growth medium, the medium is inoculated with the particular organism. Growth of the inoculum does not occur immediately, but takes a little while. This is the period of adaptation, called the lag phase. Following the lag phase, the rate of growth 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.

Chapter- 6

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.



Scanning electron microscope image of pollen.

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.

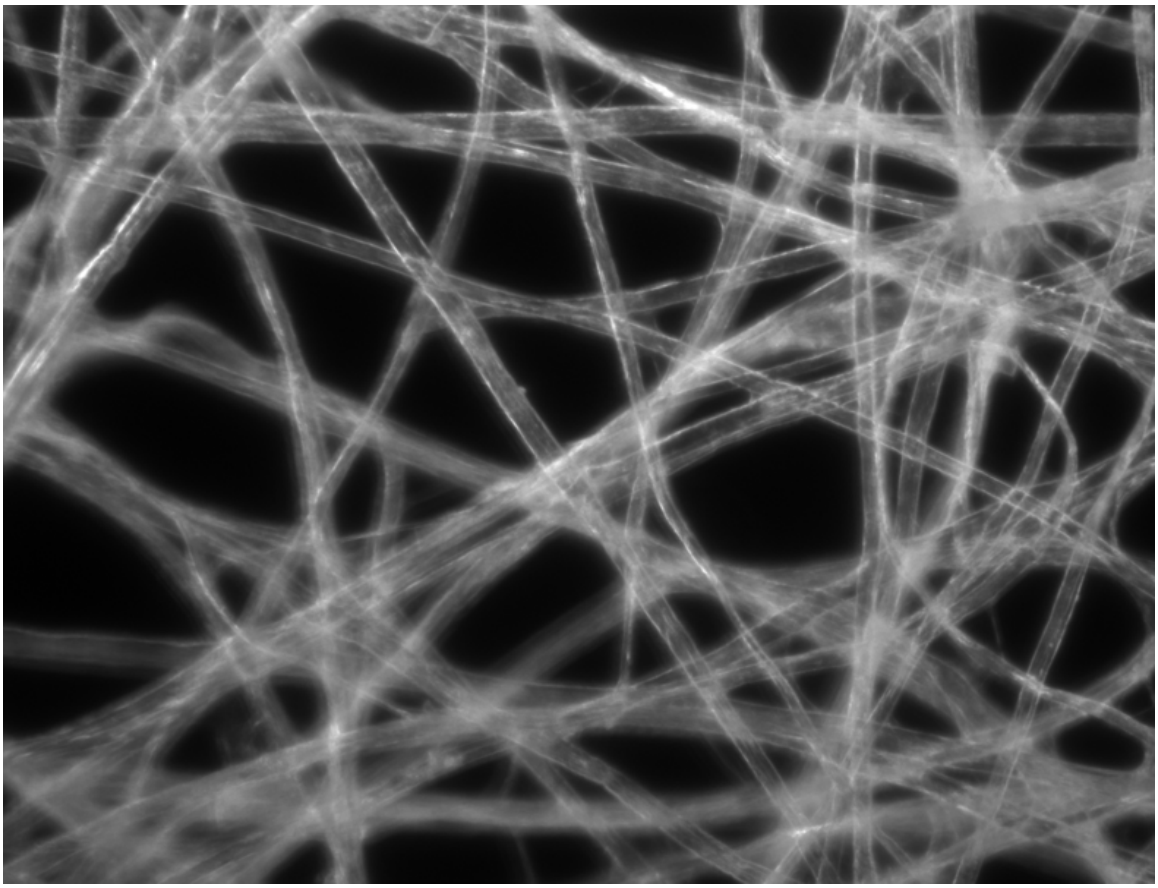
Live cells in particular generally lack sufficient contrast to be studied successfully, internal structures of the cell are colourless and transparent. The most common way to increase contrast is to stain the different structures with selective dyes, but this involves killing and fixing the sample. Staining 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).

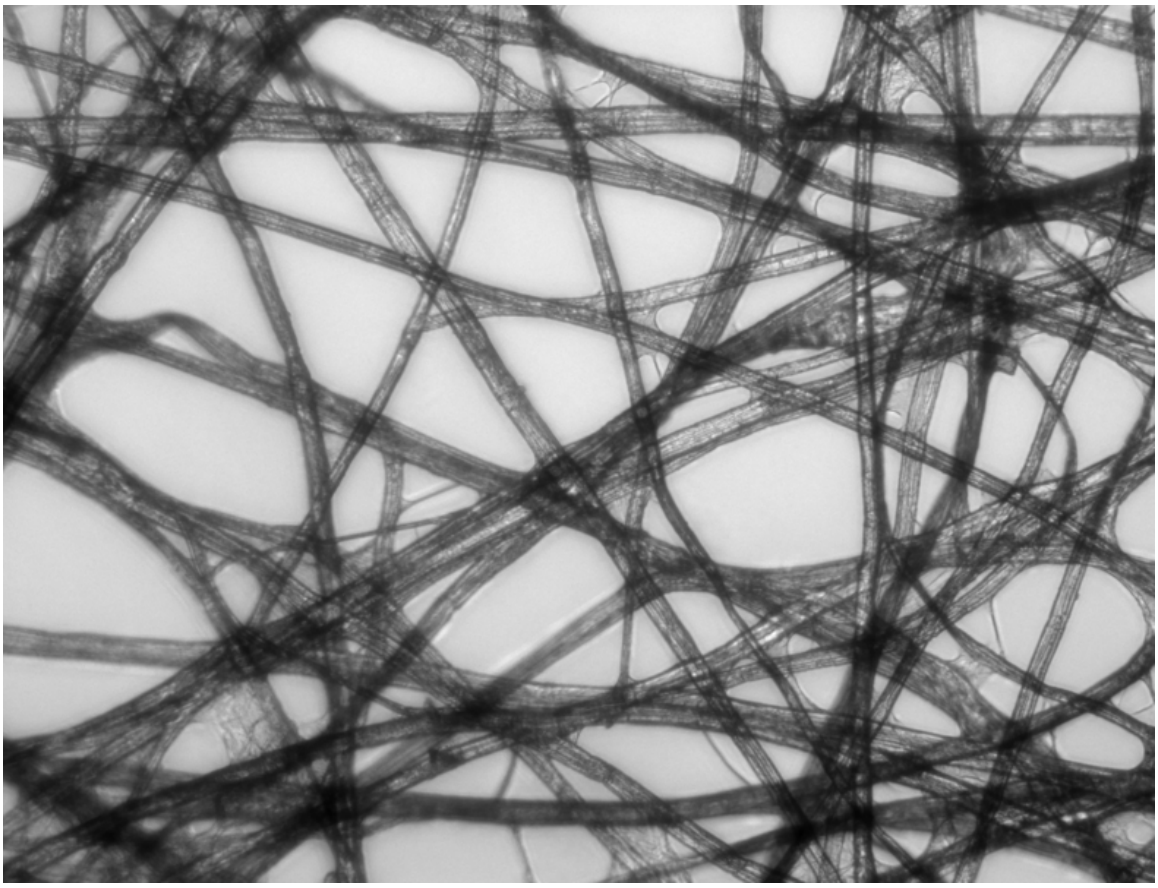
Techniques

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.

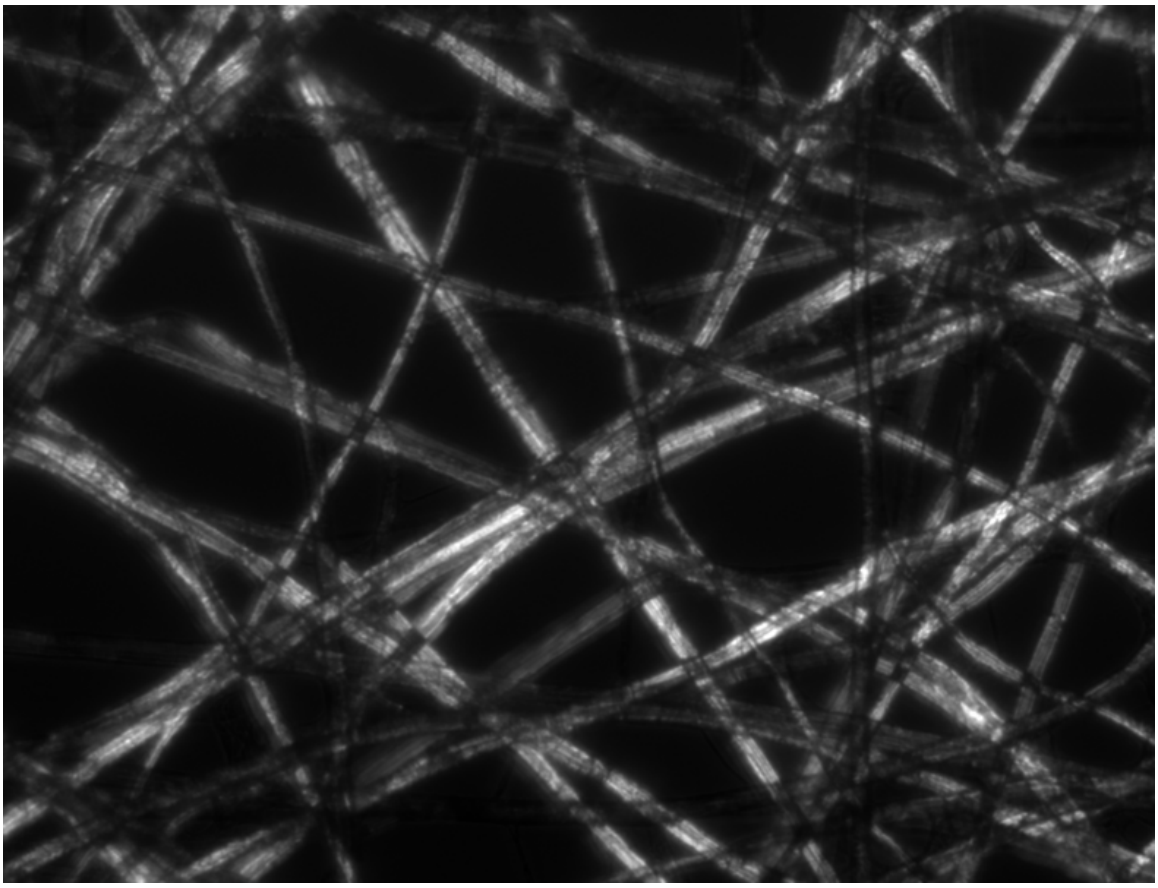
Comparison of transilumination techniques used to generate contrast in a sample of tissue paper. 1.559 µm/pixel.



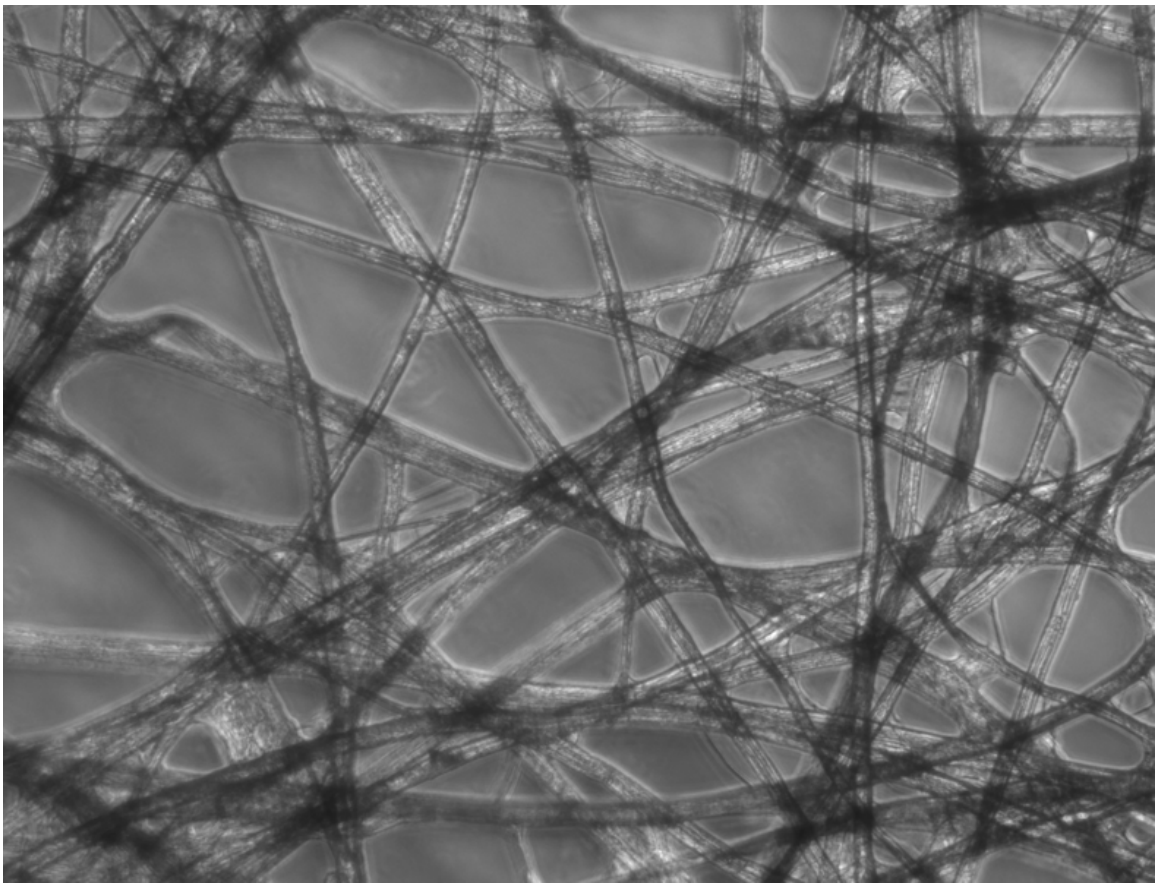
Dark field illumination, sample contrast comes from light scattered by the sample.



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



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



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

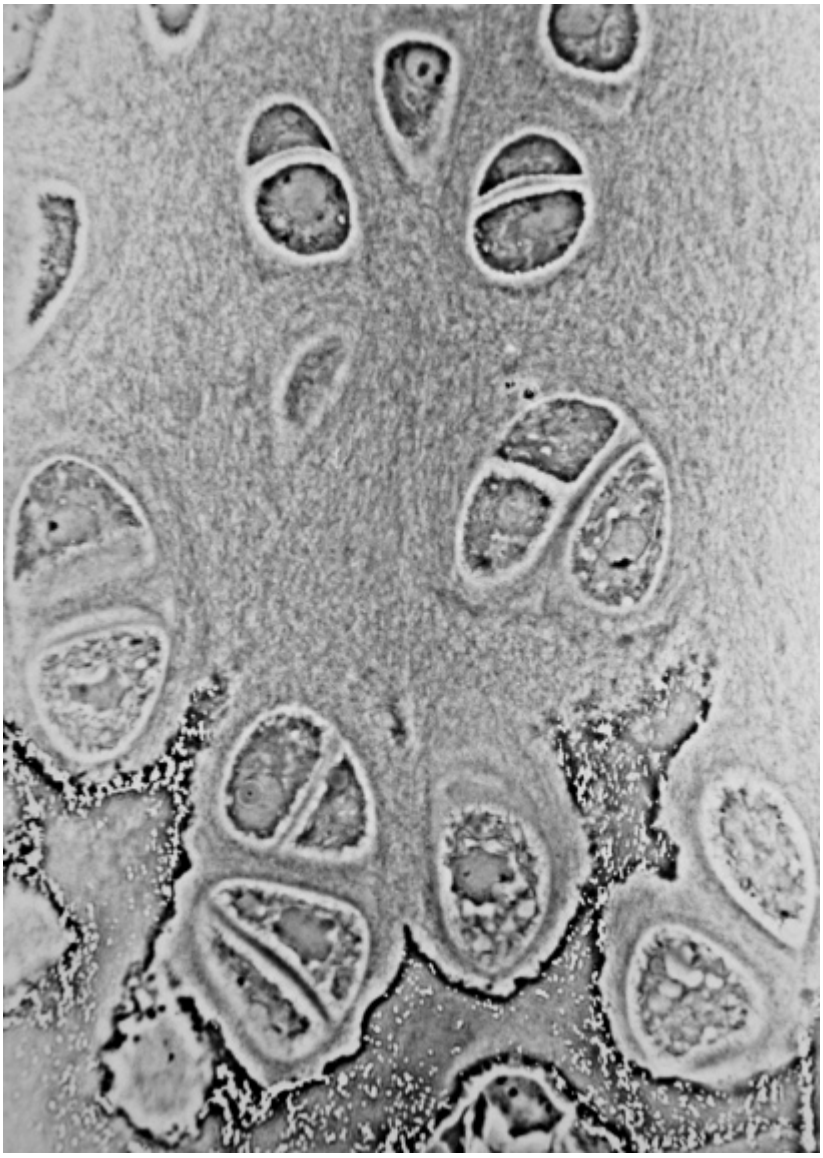
Dark field microscopy is a technique for improving the contrast of unstained, transparent specimens. Dark field illumination uses a carefully aligned light source to minimize the quantity of directly-transmitted (unscattered) light entering the image plane, collecting only the light scattered by the 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.

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.

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.

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

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

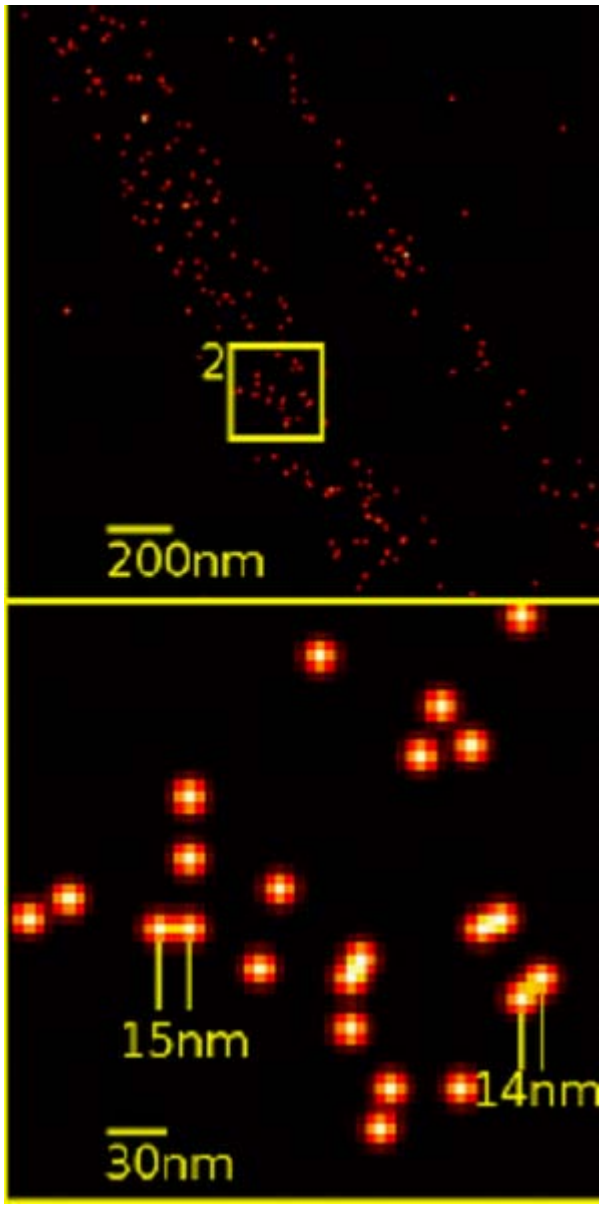
Around 1995, Christoph Cremer commenced with the development of a light microscopic process, which achieved a substantially improved size resolution of cellular nanostructures stained with a fluorescent marker. This time he employed the principle of wide field microscopy combined with structured laser 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).

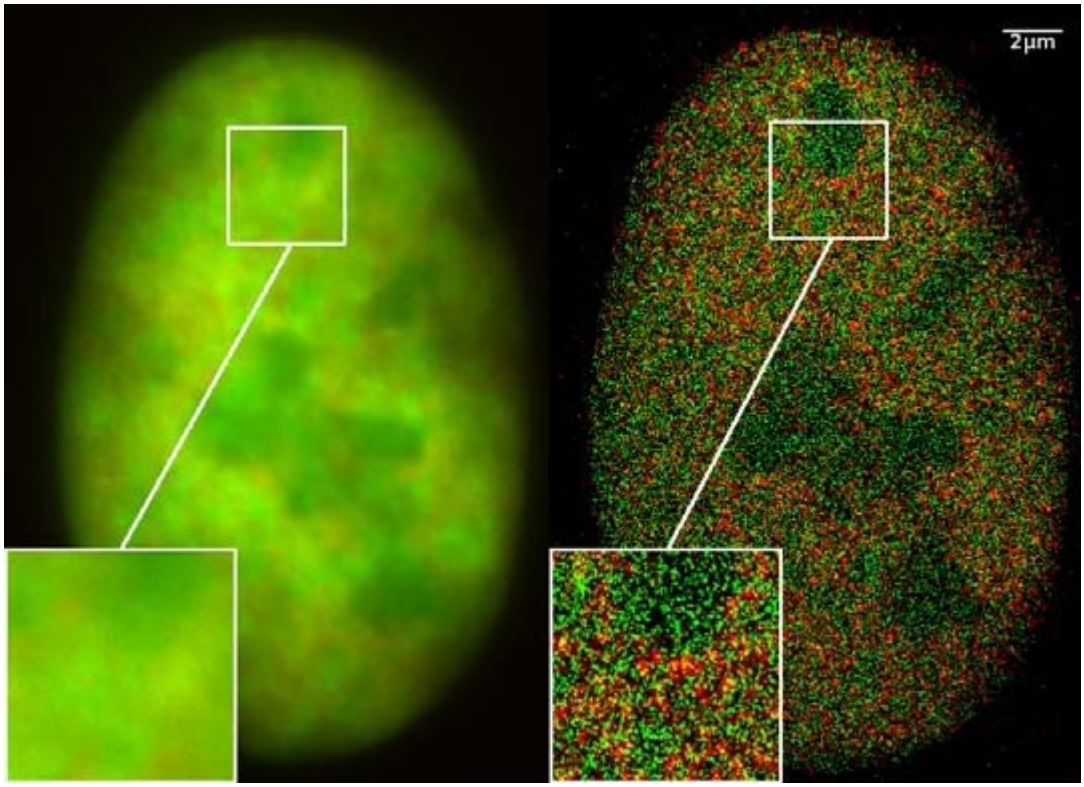
Combining SPDM and SMI, known as Vertico SMI microscopy Christoph Cremer can currently achieve a resolution of approx. 10 nm in 2D and 40 nm in 3D in wide field images of whole living cells. The Vertico SMI is currently the fastest optical 3D nanoscope for the three-dimensional structural analysis of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

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

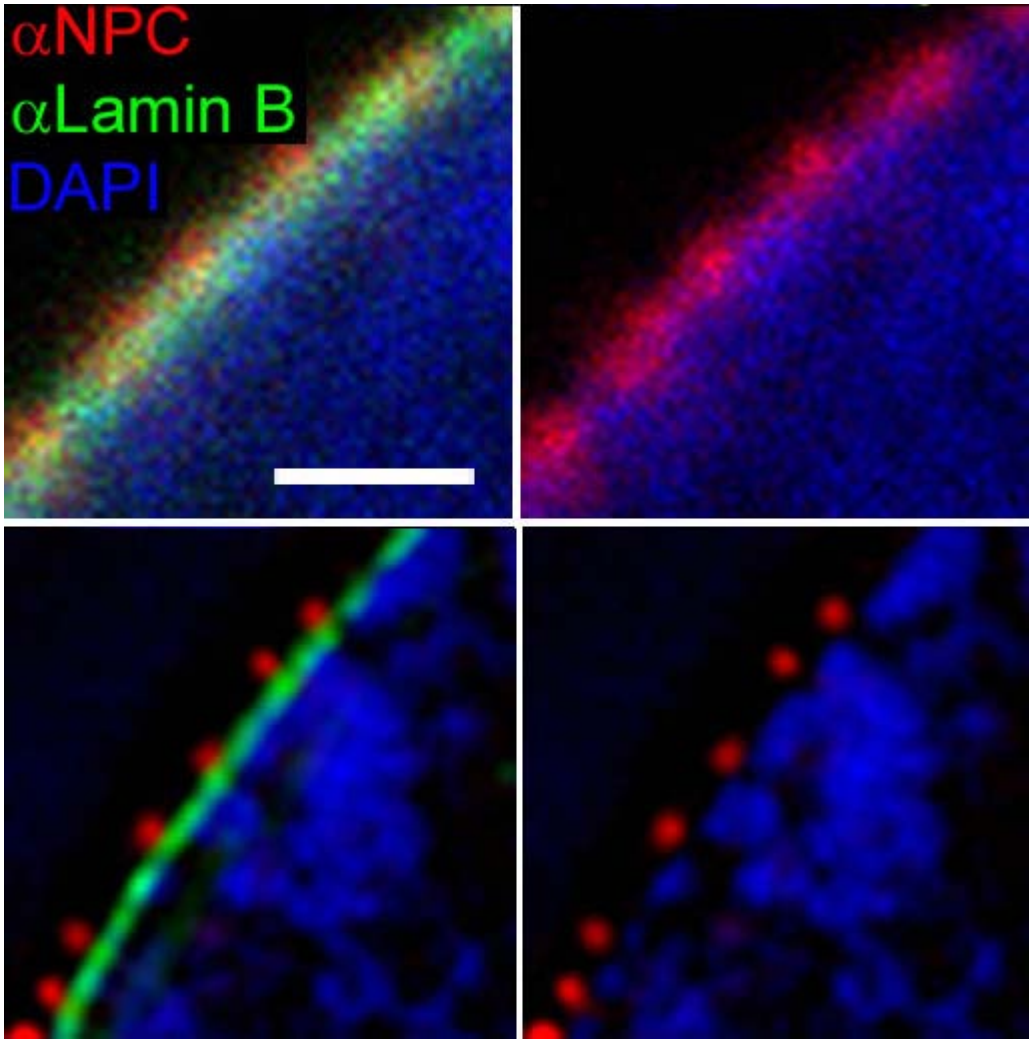
and many others have employed this analysis to localize single fluorophores to a few nanometers. This, of course, requires careful measurements and collecting *many* photons.

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).

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."

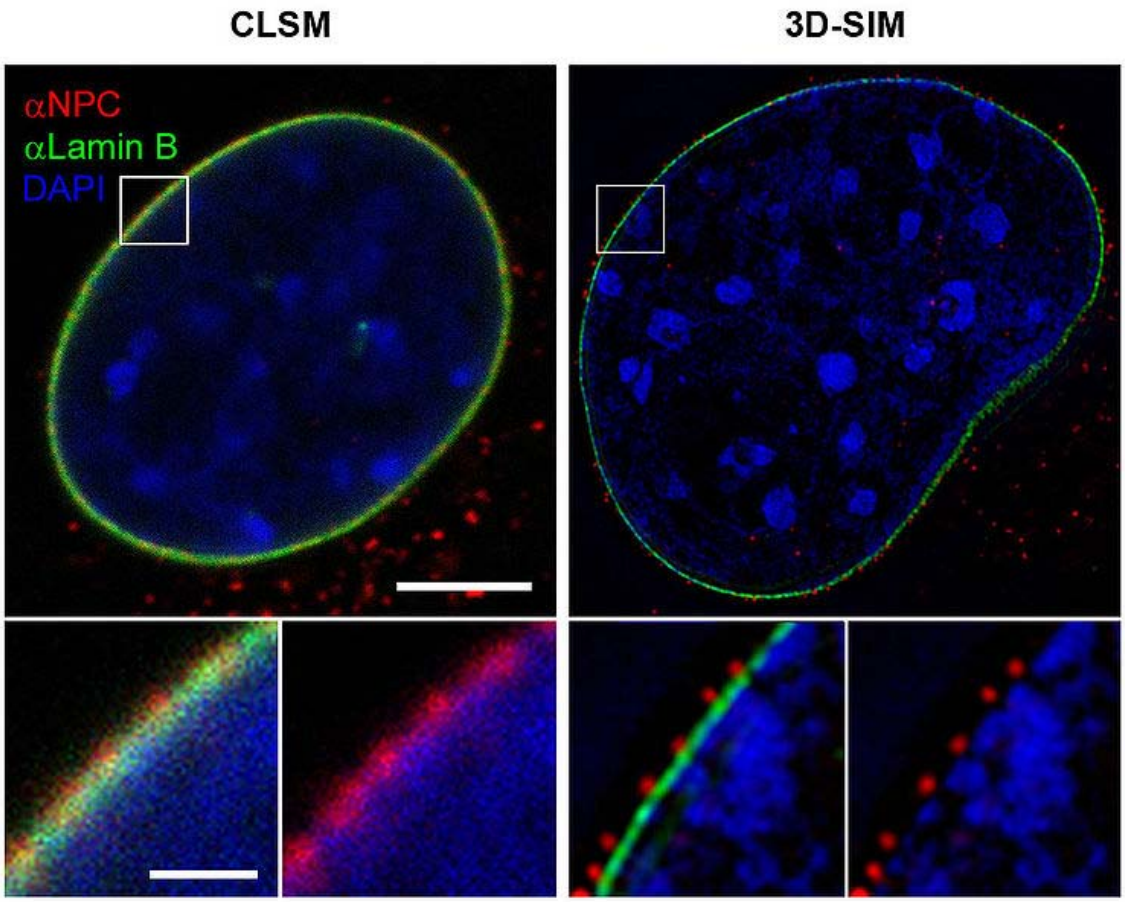
SI enhances spatial resolution by collecting information from frequency space outside the observable region. This process is done in reciprocal space: The Fourier transform (FT) of an SI image contains superimposed additional information from different areas of reciprocal space; with several frames with 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.

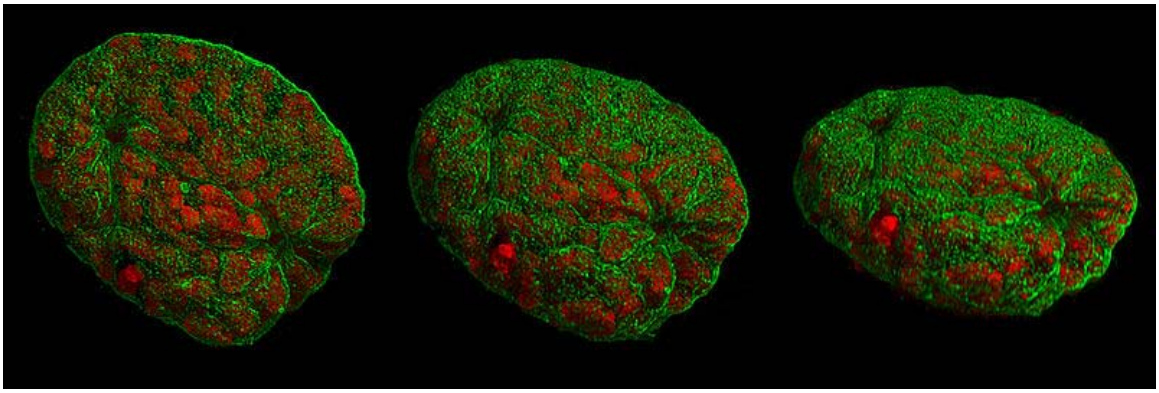
Each higher-order harmonic in the FT allows another set of images that can be used to reconstruct a larger area in reciprocal space, and thus a higher resolution. In this case, Gustafsson achieves less than 50-nm resolving power, more than five times that of the microscope in its normal configuration.

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.

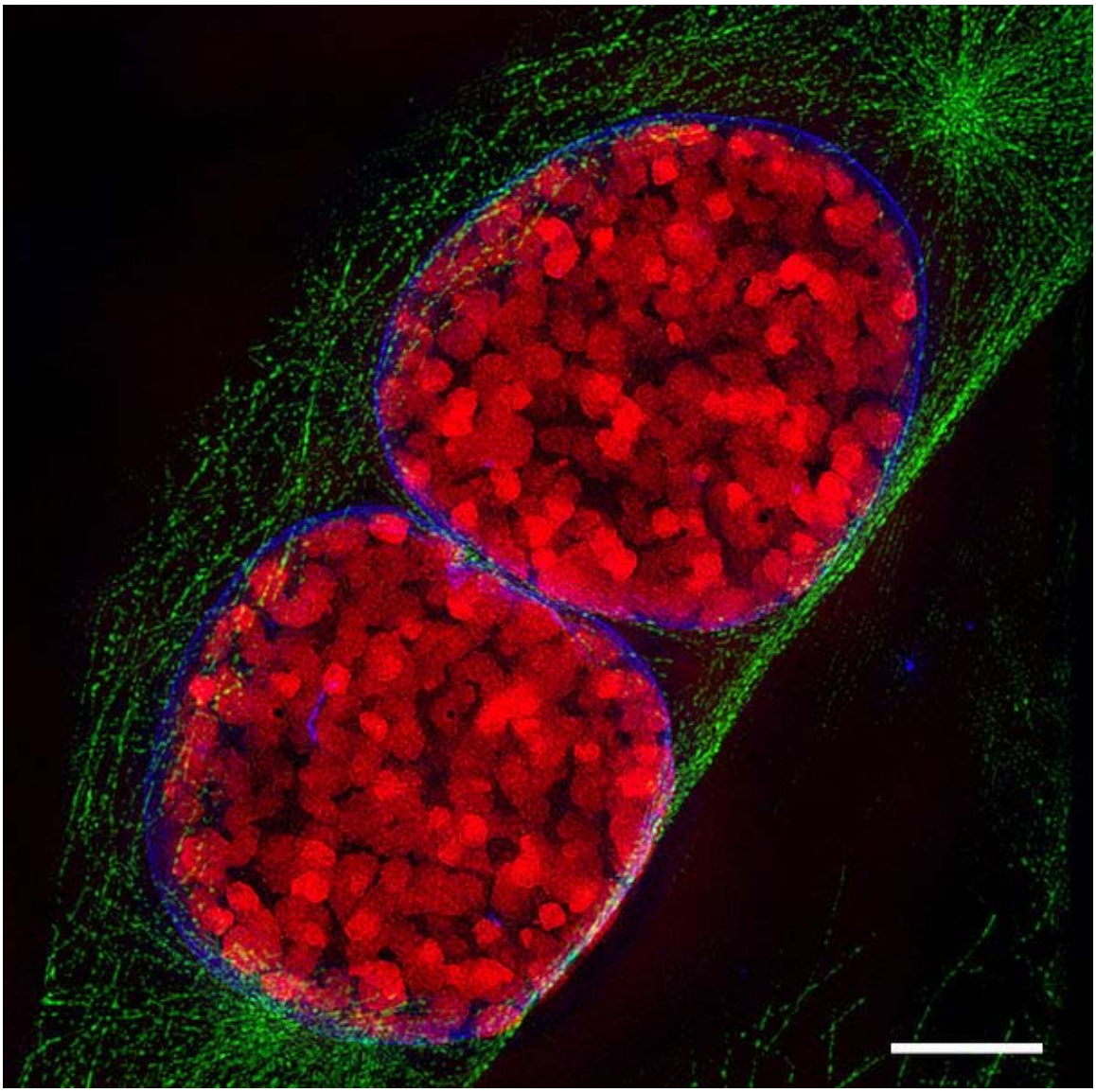
Images of cell nuclei and mitotic stages recorded with 3D-SIM Microscopy.



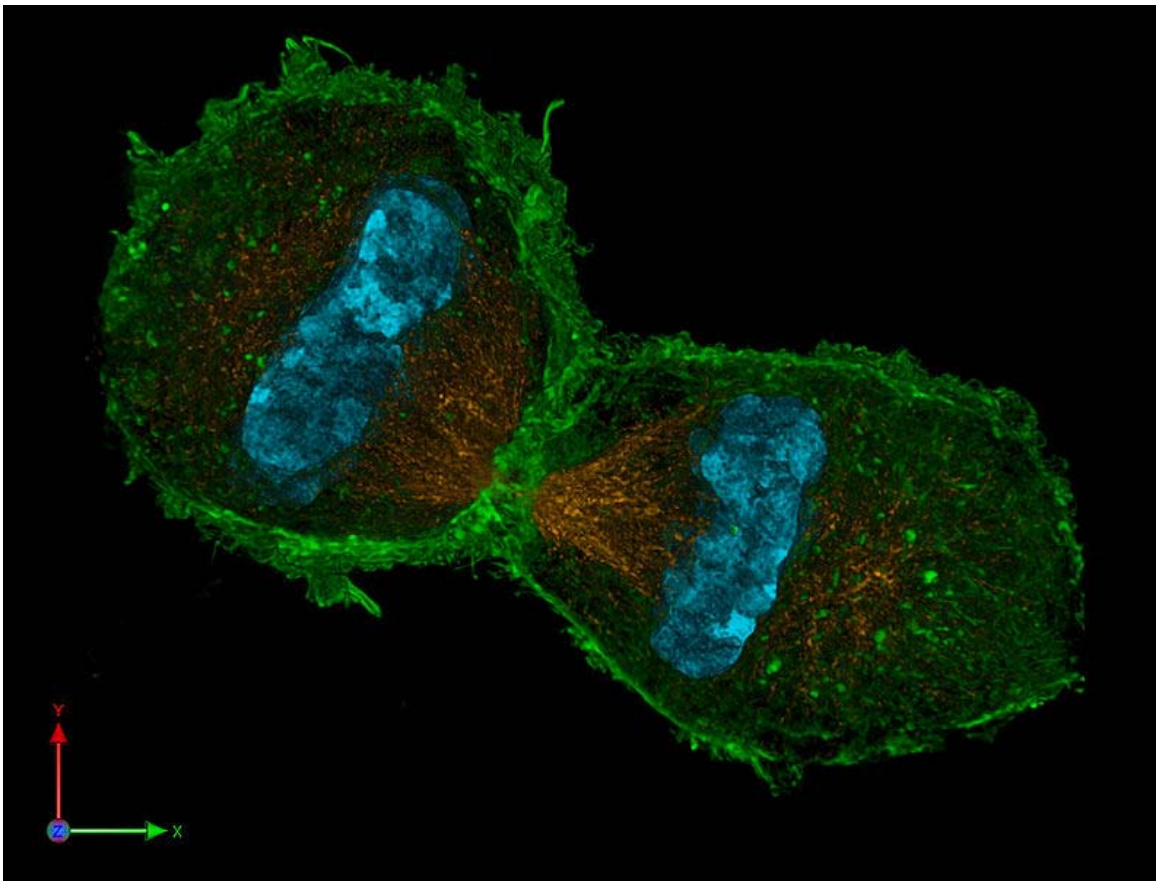
Comparison confocal microscopy - 3D-SIM



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.

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.

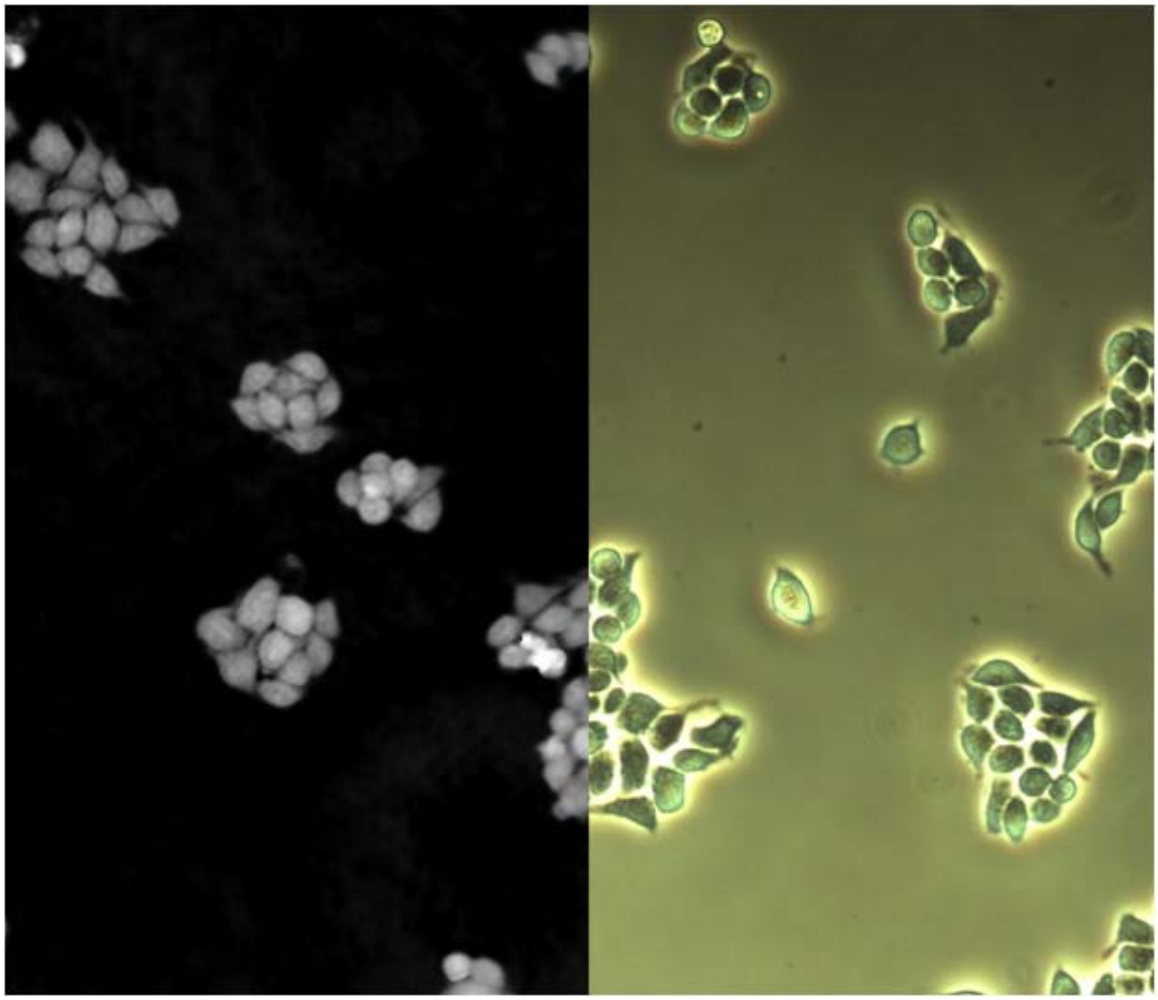
Ultrasonic force microscopy allows the local mapping of elasticity in atomic force microscopy by the application of ultrasonic vibration to the cantilever or sample. In an attempt to analyze the results of ultrasonic force microscopy in a quantitative fashion, a force-distance curve measurement is done 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.

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 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.

Chapter- 7

Electron Microscope

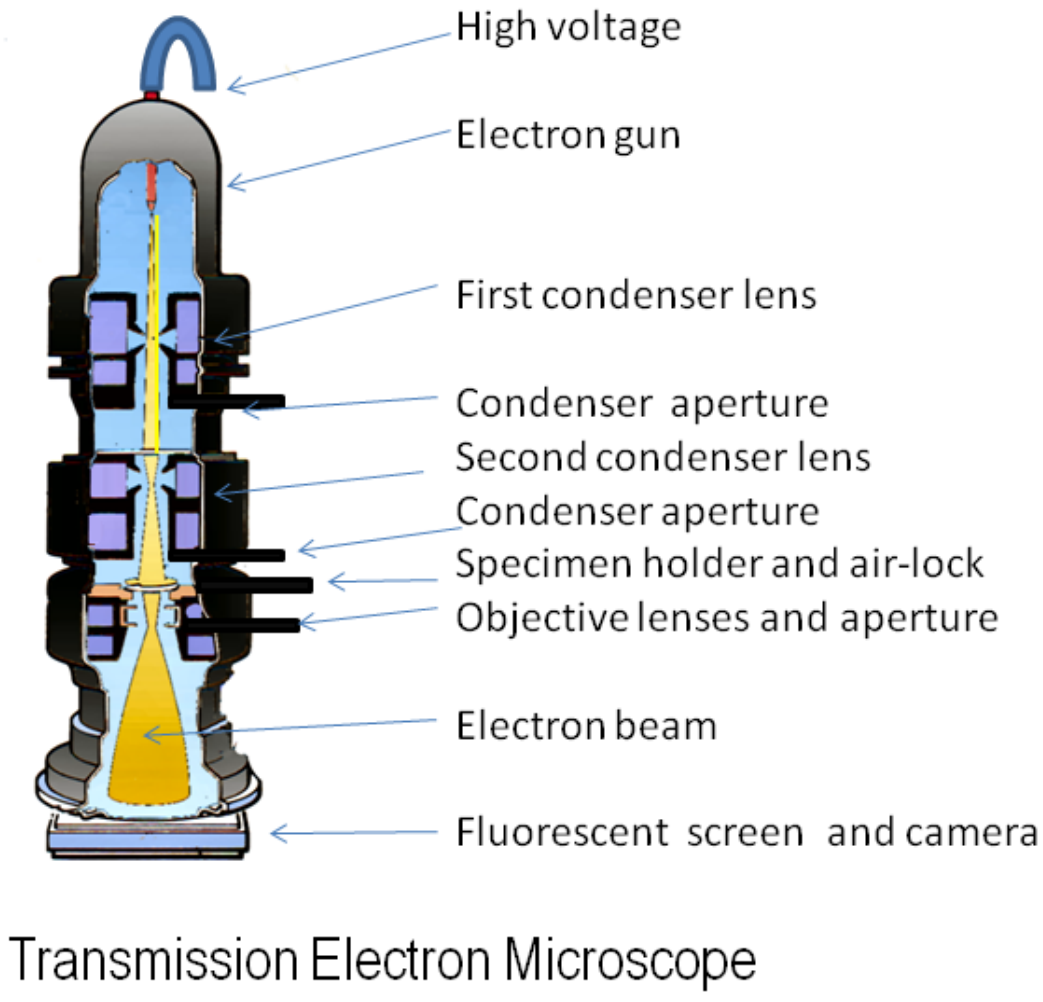


Diagram of a transmission electron microscope

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.

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:

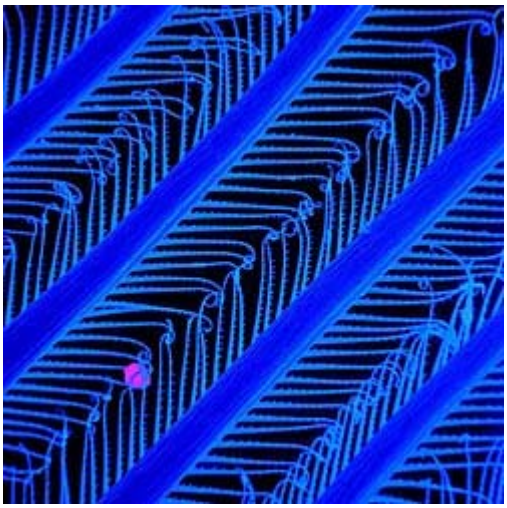
- *Chemical fixation* - for biological specimens aims to stabilize the specimen's mobile macromolecular structure by chemical crosslinking of proteins with aldehydes such as formaldehyde and glutaraldehyde, and lipids with osmium tetroxide.

- *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

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).

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.

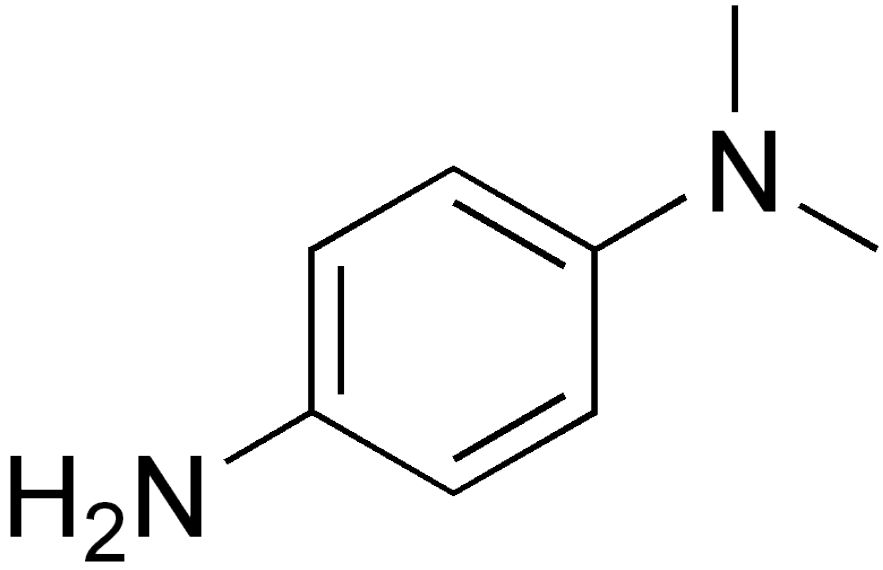
Scanning electron microscopes usually image conductive or semi-conductive materials best. Non-conductive materials can be imaged by an environmental scanning electron microscope. A common preparation technique is to coat the sample with a several-nanometer layer of conductive material, such as gold, 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.

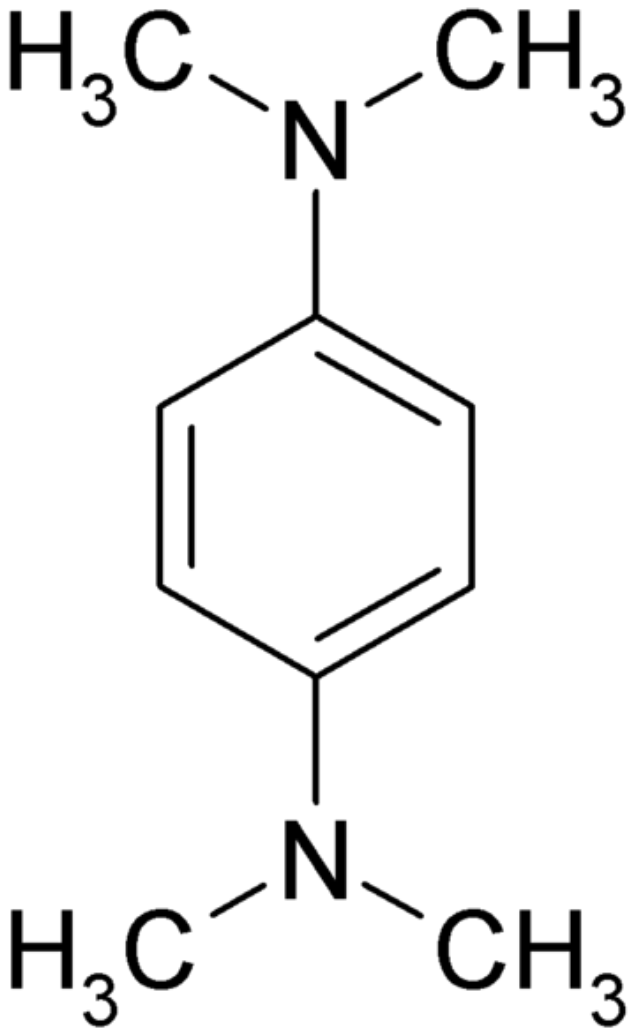
Chapter- 8

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"

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.

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.

Eukaryotic cell culture

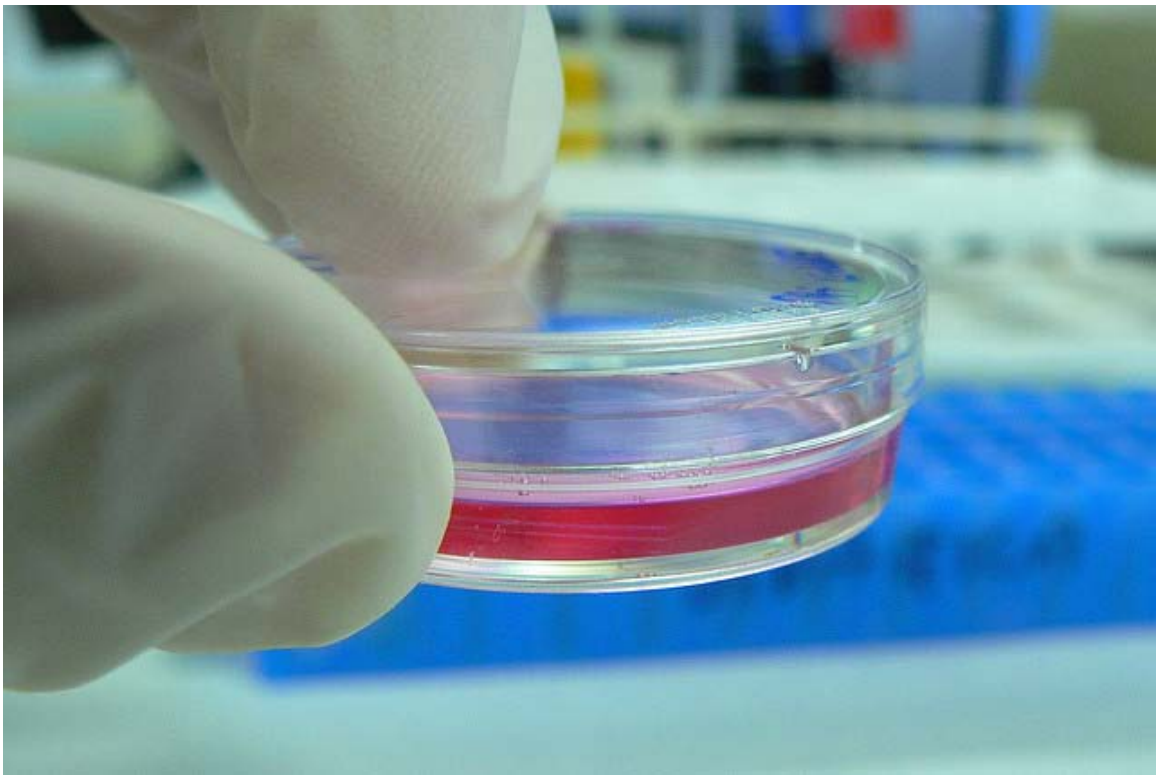
Isolation of pure cultures

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.

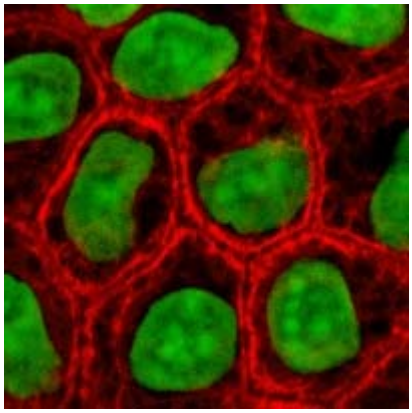
Developing pure culture techniques is crucial to the observation of the specimen in question. The most common method to isolate individual cells and produce a pure culture is to prepare a streak plate. The streak plate method is a way to physically separate the microbial population, and is done by spreading 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.

Chapter- 9

Cell Culture



Cell culture in a Petri dish



Epithelial cells in culture, stained for keratin (red) and DNA (green)

Cell culture is the complex process by which cells are grown under controlled conditions. In practice, the term "cell culture" has come to refer to the culturing of cells derived from multicellular eukaryotes, especially animal cells. However, there are also cultures of plants, fungi and microbes, including viruses, bacteria and protists. The historical development and methods of cell culture are closely interrelated to those of tissue culture and organ culture.

Animal cell culture became a common laboratory technique in the mid-1900s, but the concept of maintaining live cell lines separated from their original tissue source was discovered in the 19th century.

History

The 19th-century English physiologist Sydney Ringer developed salt solutions containing the chlorides of sodium, potassium, calcium and magnesium suitable for maintaining the beating of an isolated animal heart outside of the body. In 1885 Wilhelm Roux removed a portion of the medullary plate of an embryonic chicken and maintained it in a warm saline solution for several days, establishing the principle of tissue culture. Ross Granville Harrison, working at Johns Hopkins Medical School and then at Yale University, published results of his experiments from 1907–1910, establishing the methodology of tissue culture.

Cell culture techniques were advanced significantly in the 1940s and 1950s to support research in virology. Growing viruses in cell cultures allowed preparation of purified viruses for the manufacture of vaccines. The injectable polio vaccine developed by Jonas Salk was one of the first products mass-produced using cell culture techniques. This vaccine was made possible by the cell culture research of John Franklin Enders, Thomas Huckle Weller, and Frederick Chapman Robbins, who were awarded a Nobel Prize for their discovery of a method of growing the virus in monkey kidney cell cultures.

Concepts in mammalian cell culture

Isolation of cells

Cells can be isolated from tissues for *ex vivo* culture in several ways. Cells can be easily purified from blood, however only the white cells are capable of growth in culture. Mononuclear cells can be released from soft tissues by *enzymatic digestion* with enzymes such as collagenase, trypsin, or pronase, which break down the extracellular matrix. Alternatively, pieces of tissue can be placed in growth media, and the cells that grow out are available for culture. This method is known as *explant culture*.

Cells that are cultured directly from a subject are known as ***primary cells***. With the exception of some derived from tumors, most primary cell cultures have limited lifespan. After a certain number of population doublings (called the Hayflick limit) cells undergo the process of senescence and stop dividing, while generally retaining viability.

An established or immortalised cell line has acquired the ability to proliferate indefinitely either through random mutation or deliberate modification, such as artificial expression of the telomerase gene. There are numerous well established cell lines representative of particular cell types.

Maintaining cells in culture

Cells are grown and maintained at an appropriate temperature and gas mixture (typically, 37°C, 5% CO_2 for mammalian cells) in a cell incubator. Culture conditions vary widely for each cell type, and variation of conditions for a particular cell type can result in different phenotypes being expressed.

Aside from temperature and gas mixture, the most commonly varied factor in culture systems is the growth medium. Recipes for growth media can vary in pH, glucose concentration, growth factors, and the presence of other nutrients. The growth factors used to supplement media are often derived from animal blood, such as calf serum. One complication of these blood-derived ingredients is the potential for contamination of the culture with viruses or prions, particularly in biotechnology medical applications. Current practice is to minimize or eliminate the use of these ingredients wherever possible and use chemically defined media, but this cannot always be accomplished. Alternative strategies involve sourcing the animal blood from countries with minimum BSE/TSE risk such as Australia and New Zealand, and using purified nutrient concentrates derived from serum in place of whole animal serum for cell culture.

Plating density (number of cells per volume of culture medium) plays a critical role for some cell types. For example, a lower plating density makes granulosa cells exhibit estrogen production, while a higher plating density makes them appear as progesterone producing theca lutein cells.

Cells can be grown in *suspension* or *adherent* cultures. Some cells naturally live in suspension, without being attached to a surface, such as cells that exist in the bloodstream. There are also cell lines that have been modified to be able to survive in suspension cultures so that they can be grown to a higher density than adherent conditions would allow. Adherent cells require a surface, such as tissue culture plastic or microcarrier, which may be coated with extracellular matrix components to increase adhesion properties and provide other signals needed for growth and differentiation. Most cells derived from solid tissues are adherent. Another type of adherent culture is *organotypic culture* which involves growing cells in a three-dimensional environment as opposed to two-dimensional culture dishes. This 3D culture system is biochemically and physiologically more similar to *in vivo* tissue, but is technically challenging to maintain because of many factors (e.g. diffusion).

Cell line cross-contamination

Cell line cross-contamination can be a problem for scientists working with cultured cells. Studies suggest that anywhere from 15–20% of the time, cells used in experiments have been misidentified or contaminated with another cell line. Problems with cell line cross contamination have even been detected in lines from the NCI-60 panel, which are used routinely for drug-screening studies. Major cell line repositories including the American Type Culture Collection (ATCC) and the German Collection of Microorganisms and Cell Cultures (DSMZ) have received cell line submissions from researchers that were misidentified by the researcher. Such contamination poses a problem for the quality of research produced using cell culture lines, and the major repositories are now authenticating all cell line submissions. ATCC uses short tandem repeat (STR) DNA fingerprinting to authenticate its cell lines.

To address this problem of cell line cross-contamination, researchers are encouraged to authenticate their cell lines at an early passage to establish the identity of the cell line. Authentication should be repeated before freezing cell line stocks, every two months during active culturing and before any publication of research data generated using the cell lines. There are many methods for identifying cell lines including isoenzyme analysis, human lymphocyte antigen (HLA) typing, Chromosomal analysis, Karyotyping, Morphology and STR analysis.

One significant cell-line cross contaminant is the immortal HeLa cell line.

Manipulation of cultured cells

As cells generally continue to divide in culture, they generally grow to fill the available area or volume. This can generate several issues:

- Nutrient depletion in the growth media
- Accumulation of apoptotic/necrotic (dead) cells.
- Cell-to-cell contact can stimulate cell cycle arrest, causing cells to stop dividing known as contact inhibition or senescence.

- Cell-to-cell contact can stimulate cellular differentiation.

Among the common manipulations carried out on culture cells are media changes, passaging cells, and transfecting cells. These are generally performed using tissue culture methods that rely on sterile technique. Sterile technique aims to avoid contamination with bacteria, yeast, or other cell lines. Manipulations are typically carried out in a biosafety hood or laminar flow cabinet to exclude contaminating micro-organisms. Antibiotics (e.g. penicillin and streptomycin) and antifungals (e.g. Amphotericin B) can also be added to the growth media.

As cells undergo metabolic processes, acid is produced and the pH decreases. Often, a pH indicator is added to the medium in order to measure nutrient depletion.

Media changes

In the case of adherent cultures, the media can be removed directly by aspiration and replaced.

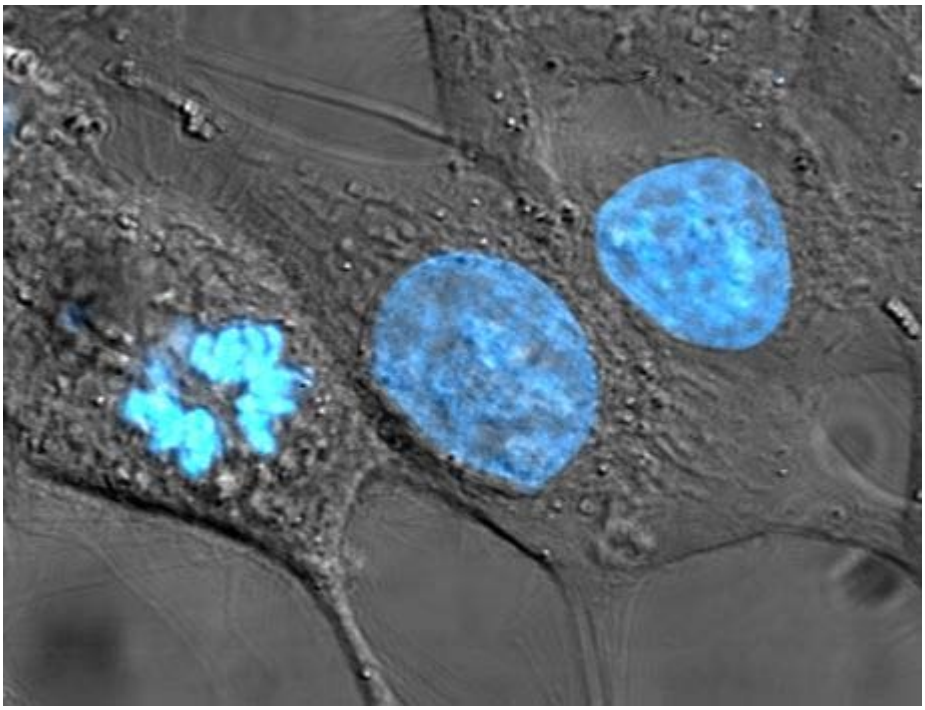
Passaging cells

Passaging (also known as subculture or splitting cells) involves transferring a small number of cells into a new vessel. Cells can be cultured for a longer time if they are split regularly, as it avoids the senescence associated with prolonged high cell density. Suspension cultures are easily passaged with a small amount of culture containing a few cells diluted in a larger volume of fresh media. For adherent cultures, cells first need to be detached; this is commonly done with a mixture of trypsin-EDTA, however other enzyme mixes are now available for this purpose. A small number of detached cells can then be used to seed a new culture.

Transfection and transduction

Another common method for manipulating cells involves the introduction of foreign DNA by transfection. This is often performed to cause cells to express a protein of interest. More recently, the transfection of RNAi constructs have been realized as a convenient mechanism for suppressing the expression of a particular gene/protein. DNA can also be inserted into cells using viruses, in methods referred to as transduction, infection or transformation. Viruses, as parasitic agents, are well suited to introducing DNA into cells, as this is a part of their normal course of reproduction.

Established human cell lines



Cultured HeLa cells have been stained with Hoechst turning their nuclei blue, and are one of the earliest human cell lines descended from Henrietta Lacks, who died of cervical cancer from which these cells originated.

Cell lines that originate with humans have been somewhat controversial in bioethics, as they may outlive their parent organism and later be used in the discovery of lucrative medical treatments. In the pioneering decision in this area, the Supreme Court of California held in *Moore v. Regents of the University of California* that human patients have no property rights in cell lines derived from organs removed with their consent.

Generation of hybridomas

It is possible to fuse normal cells with an immortalised cell line. This method is used to produce monoclonal antibodies. In brief, lymphocytes isolated from the spleen (or possibly blood) of an immunised animal are combined with an immortal myeloma cell line (B cell lineage) to produce a hybridoma which has the antibody specificity of the primary lymphoctye and the immortality of the myeloma. Selective growth medium (HA or HAT) is used to select against unfused myeloma cells; primary lymphoctyes die quickly in culture and only the fused cells survive. These are screened for production of the required antibody, generally in pools to start with and then after single cloning.

Applications of cell culture

Mass culture of animal cell lines is fundamental to the manufacture of viral vaccines and other products of biotechnology

Biological products produced by recombinant DNA (rDNA) technology in animal cell cultures include enzymes, synthetic hormones, immunobiologicals (monoclonal antibodies, interleukins, lymphokines), and anticancer agents. Although many simpler proteins can be produced using rDNA in bacterial cultures, more complex proteins that are glycosylated (carbohydrate-modified) currently must be made in animal cells. An important example of such a complex protein is the hormone erythropoietin. The cost of growing mammalian cell cultures is high, so research is underway to produce such complex proteins in insect cells or in higher plants, use of single embryonic cell and somatic embryos as a source for direct gene transfer via particle bombardment, transit gene expression and confocal microscopy observation is one of its applications. It also offers to confirm single cell origin of somatic embryos and the asymmetry of the first cell division, which starts the process. -

Tissue culture and engineering

Cell culture is a fundamental component of tissue culture and tissue engineering, as it establishes the basics of growing and maintaining cells *ex vivo*. The major application of human cell culture is in stem cell industry where mesenchymal stem cells can be cultured and cryopreserved for future use.

Vaccines

Vaccines for polio, measles, mumps, rubella, and chickenpox are currently made in cell cultures. Due to the H5N1 pandemic threat, research into using cell culture for influenza vaccines is being funded by the United States government. Novel ideas in the field include recombinant DNA-based vaccines, such as one made using human adenovirus (a common cold virus) as a vector, , such as adjuvants.

Culture of non-mammalian cells

Plant cell culture methods

Plant cell cultures are typically grown as cell suspension cultures in liquid medium or as callus cultures on solid medium. The culturing of undifferentiated plant cells and calli requires the proper balance of the plant growth hormones auxin and cytokinin.

Bacterial and yeast culture methods

For bacteria and yeast, small quantities of cells are usually grown on a solid support that contains nutrients embedded in it, usually a gel such as agar, while large-scale cultures are grown with the cells suspended in a nutrient broth.

Viral culture methods

The culture of viruses requires the culture of cells of mammalian, plant, fungal or bacterial origin as hosts for the growth and replication of the virus. Whole wild type

viruses, recombinant viruses or viral products may be generated in cell types other than their natural hosts under the right conditions. Depending on the species of the virus, infection and viral replication may result in host cell lysis and formation of a viral plaque.

Common cell lines

Human cell lines

- National Cancer Institute's 60 cancer cell lines

- DU145 (Prostate cancer)
- Lncap (Prostate cancer)
- MCF-7 (breast cancer)
- MDA-MB-438 (breast cancer)
- PC3 (Prostate cancer)
- T47D (breast cancer)
- THP-1 (acute myeloid leukemia)
- U87 (glioblastoma)
- SHSY5Y Human neuroblastoma cells, cloned from a myeloma
- Saos-2 cells (bone cancer)

Primate cell lines

- Vero (African green monkey *Chlorocebus* kidney epithelial cell line initiated 1962)

Rat tumor cell lines

- GH3 (pituitary tumor)
- PC12 (pheochromocytoma)

Mouse cell lines

- MC3T3 (embryonic calvarial)

Plant cell lines

- Tobacco BY-2 cells (kept as cell suspension culture, they are model system of plant cell)

Other species cell lines

- zebrafish ZF4 and AB9 cells.
- *Madin-Darby Canine Kidney (MDCK)* epithelial cell line
- Xenopus A6 kidney epithelial cells.

List of cell lines

Cell line	Meaning	Organism	Origin tissue	Morphology	Link
293-T		Human	Kidney (embryonic)		Derivative of HEK 293ECACC
3T3 cells	"3-day transfer, inoculum 3 x 10^5 cells"	Mouse	Embryonic fibroblast		Also known as NIH 3T3 ECACC
721		Human	Melanoma		
9L		Rat	Glioblastoma		
A2780		Human	Ovary	Ovarian Cancer	ECACC
A2780ADR		Human	Ovary	Adriamycin-resistant derivative	ECACC
A2780cis		Human	Ovary	Cisplatin-resistant derivative	ECACC
A172		Human	glioblastoma	malignant glioma	ECACC
A20		Murine	B lymphoma	B lymphocyte	
A253		Human	Head and neck carcinoma	submandibular duct	
A431		Human	Skin epithelium	squamous carcinoma	ECACCCell Line Data Base
A-549		Human	Lungcarcinoma	Epithelium	DSMZECACC
ALC		Murine	bone marrow	Stroma	PubMed
B16		Murine	Melanoma		ECCAC
B35		Rat	Neuroblastoma		ATCC
BCP-1 cells		Human	PBMC	HIV+ Lymphoma	ATCC
BEAS-2B	Bronchial epithelium + Adenovirus 12-SV40 virus hybrid (Ad12SV40)	Human	Lung	Epithelial	ATCC
bEnd.3	*Brain endothelial*	Mouse	Brain / Cerebral cortex	Endothelium	ATCC
BHK-21	"Baby Hamster Kidney Fibroblast cells"	Hamster	Kidney	fibroblast	ECACCOlympus
BR 293		Human	Breast	Breast cancer	
BxPC3	Biopsy xenograph of pancreatic carcinoma line 3	Human	pancreatic adenocarcinoma	Epithelial	ATCC
C3H-10T1/2		Mouse	Embryonic mesenchymal cell line		ECACC
C6/36		Asian tiger mosquito	larval tissue		ECACC
Cal-27		Human	Tongue	squamous cell	

				carcinoma	
CHO	*Chinese hamster ovary*	hamster	Ovary	Epithelium	ECACCICLC
COR-L23		Human	Lung		ECACC
COR-L23/CPR		Human	Lung		ECACC
COR-L23/5010		Human	Lung		ECACC
COR-L23/R23		Human	Lung	Epithelial	ECACC
COS-7	*Cercopithecus aethiops, origin-defective SV-40*	Ape - *Cercopithecus aethiops* (Chlorocebus)	Kidney	fibroblast	ECACCATCC
COV-434		Human	Ovary	Metastatic granulosa cell carcinoma	ECACC
CML T1	*Chronic Myelod Leukaemia T-lymphocyte 1*	Human	CML acute phase	T cell leukaemia	Blood
CMT	*canine mammary tumor*	Dog	Mammary gland	Epithelium	
CT26		Murine	Colorectal Carcinoma	Colon	
D17		canine	osteosarcoma		ECACC
DH82		canine	histiocytosis	monocyte/macrophage	ECACC J Vir Meth
DU145		Human	Androgen insensitive carcinoma	Prostate	PubMed
DuCaP	Dura mater Cancer of the Prostate	Human	Metastatic Prostate Cancer	Epithelial	EAC { Ehrlich Ascites Carcinoma } mice
EL4		Mouse		T cell leukaemia	ECACC
EM2		Human	CML blast crisis	Ph+ CML line	Cell Line Data Base
EM3		Human	CML blast crisis	Ph+ CML line	Cell Line Data Base
EMT6/ AR1		Mouse	Breast	Epithelial-like	ECACC
EMT6/ AR10.0		Mouse	Breast	Epithelial-like	ECACC
FM3		Human	Metastatic lymph node	melanoma	
H1299		Human	Lung	Lung cancer	

H69		Human	Lung		ECACC
HB54		hybridoma	hybridoma	secretes L243 mAb (against HLA-DR)	Human Immunology
HB55		hybridoma	hybridoma	secretes MA2.1 mAb (against HLA-A2 and HLA-B17)	Journal of Immunology
HCA2		Human	fibroblast		Journal of General Virology
HEK-293	*Human embryonic kidney*	Human	Kidney (embryonic)	Epithelium	ATCC
HeLa	*Henrietta Lacks*	Human	Cervical cancer	Epithelium	DSMZECACC
Hepa1c1c7	clone 7 of clone 1 hepatoma line 1	Mouse	Hepatoma	Epithelial	ECACC ATCC
HL-60	*Human leukemia*	Human	Myeloblast	bloodcells	ECACCDSMZ
HMEC	*Human mammary epithelial cell*	Human		Epithelium	ECACC
HT-29		Human	Colon epithelium	Adenocarcinoma	ECACC Cell Line Data Base
Jurkat		Human	T-Cell-Leukemia	white blood cells	ECACC DSMZ
JY cells		Human	Lymphoblastoid	EBV immortalised B cell	
K562 cells		Human	Lymphoblastoid	CML blast crisis	ECACC
Ku812		Human	Lymphoblastoid	erythroleukemia	ECACC LGCstandards
KCL22		Human	Lymphoblastoid	CML	
KG1		Human	Lymphoblastoid	AML	
KYO1	Kyoto 1	Human	Lymphoblastoid	CML	DSMZ
LNCap	Lymph node Cancer of the Prostate	Human	prostatic adenocarcinoma	Epithelial	ECACCATCC
Ma-Mel 1, 2, 3....48		Human		a range of melanoma cell lines	
MC-38		Mouse		Adenocarcinoma	
MCF-7	*Michigan Cancer Foundation-7*	Human	Mammary gland	Invasive breast ductal carcinoma	ER+, PR+
MCF-10A	*Michigan Cancer Foundation*	Human	mammary gland	Epithelium	ATCC
MDA-MB-	M.D. Anderson	Human	Breast	Cancer	ECACC

231	- Metastatic Breast				
MDA-MB-468	M.D. Anderson - Metastatic Breast	Human	Breast	Cancer	ECACC
MDA-MB-435	M.D. Anderson - Metastatic Breast	Human	Breast	melanoma or carcinoma (disputed)	Cambridge Pathology ECACC
MDCK II	*Madin Darby canine kidney*	Dog	Kidney	Epithelium	ECACC ATCC
MDCK II	*Madin Darby canine kidney*	Dog	Kidney	Epithelium	ATCC
MOR/0.2R		Human	Lung		ECACC
MONO-MAC 6		Human	WBC	myeloid metaplasic AML	Cell Line Data Base
MTD-1A		Mouse		Epithelium	
MyEnd	*Myocardial endothelial*	Mouse		Endothelium	
NCI-H69/CPR		Human	Lung		ECACC
NCI-H69/LX10		Human	Lung		ECACC
NCI-H69/LX20		Human	Lung		ECACC
NCI-H69/LX4		Human	Lung		ECACC
NIH-3T3	*NIH, 3-day transfer, inoculum 3 x 10^5 cells*	Mouse	embryo	fibroblast	ECACCATCC
NALM-1			peripheral blood	blast-crisis CML	Cancer Genetics and Cytogenetics
NW-145				Melanoma	ESTDAB
OPCN / OPCT cell lines	Onyvax Prostate Cancer....			Range of prostate tumour lines	Asterand
Peer		Human	T cell leukemia		DSMZ
PNT-1A / PNT 2				Prostate tumour lines	ECACC
RenCa	Renal Carcinoma	Mouse		renal carcinoma	
RIN-5F		Mouse	Pancreas		
RMA/ RMAS		Mouse		T cell tumour	
Saos-2 cells		Human		Osteosarcoma	ECACC
Sf-9	*Spodoptera frugiperda*	insect - *Spodoptera frugiperda* (moth)	Ovary		DSMZECACC

SkBr3		Human		Breast carcinoma	
T2		Human		T cell leukemia/B cell line hybridoma	DSMZ
T-47D		Human	Mammary gland	ductal carcinoma	
T84		Human	colorectal Carcinoma / Lungmetastasis	Epithelium	ECACCATCC
THP1 cell line		Human	Monocyte	AML	ECACC
U373		Human	Glioblastoma-astrocytoma	Epithelium	
U87		Human	glioblastoma-astrocytoma	Epithelial-like	Abcam
U937		Human	Leukaemic monocytic lymphoma		ECACC
VCaP	Vertebra Prostate Cancer	Human	Metastatic prostate cancer	Epithelial	ECACC ATCC
Vero cells	'Vera Reno' ('Green kidney') / 'Vero' ('truth')	African Green Monkey	Kidney epithelium		ECACC
WM39		Human	skin	Primary melanoma	
WT-49		Human	Lymphoblastoid		
X63		Mouse	Melanoma		
YAC-1		Mouse	Lymphoma		Cell Line Data Base ECACC
YAR		Human	B-cell	EBV transofrmed	Human Immunology

Note: this list is a sample of available cell lines, and is not comprehensive

Chapter- 10

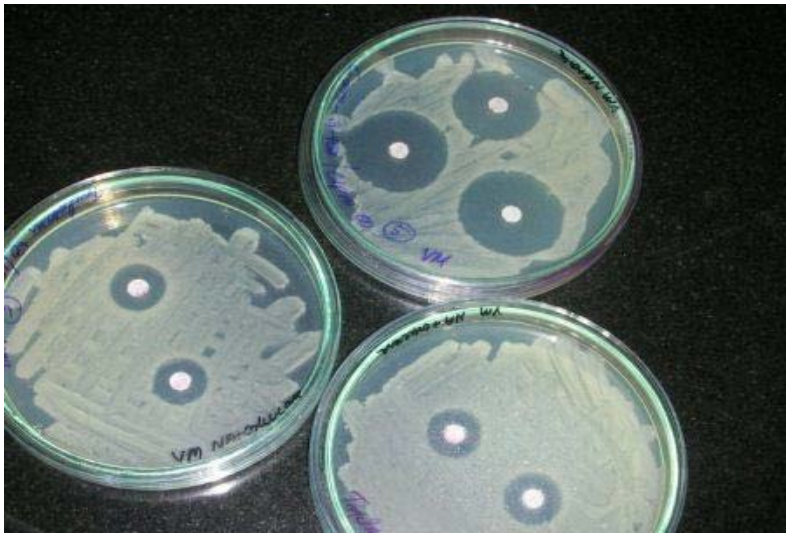
Kirby-Bauer Antibiotic Testing, Miles and Misra Method, Streaking & Replica Plating

Kirby-Bauer antibiotic testing

In Kirby-Bauer testing, white wafers containing antibiotics are placed on a plate of bacteria. Circles of poor bacterial growth surround some wafers indicating susceptibility to the antibiotic.

Kirby-Bauer antibiotic testing (**KB testing** or **disk diffusion antibiotic sensitivity testing**) is a test which uses antibiotic-impregnated wafers to test whether particular bacteria are susceptible to specific antibiotics. A known quantity of bacteria are grown on agar plates in the presence of thin wafers containing relevant antibiotics. If the bacteria are susceptible to a particular antibiotic, an area of clearing surrounds the wafer where bacteria are not capable of growing (called a zone of inhibition).

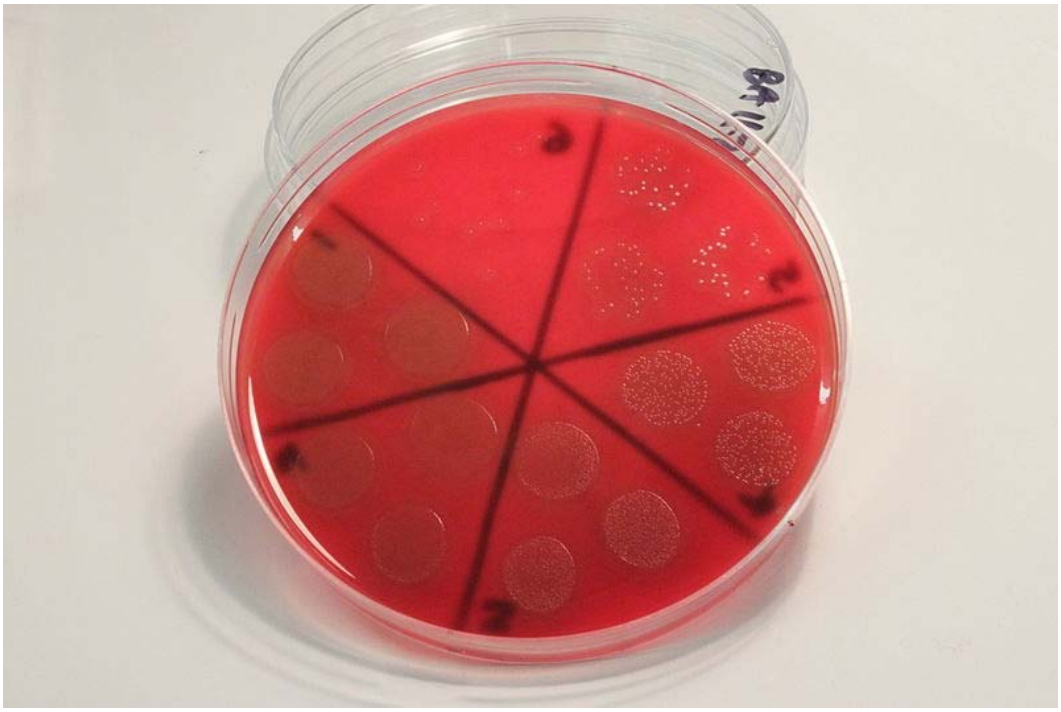
Placing antibiotic disk on agar plates streaked with bacterial suspension(Inside a Laminar Airflow system)



The zones of inhibition using different concentrations of Ciprofloxacin

This along with the rate of antibiotic diffusion are used to estimate the bacteria's sensitivity to that particular antibiotic. In general, larger zones correlate with smaller minimum inhibitory concentration (MIC) of antibiotic for that bacteria. This information can be used to choose appropriate antibiotics to combat a particular infection.

Miles and Misra method



Miles & Misra method. Dilutions of *S.pneumoniae* on blood agar plate.

The Miles and Misra Method (or surface variable count) is a technique used in Microbiology to determine the number of colony forming units in a bacterial suspension or homogenate. The technique was first described in 1938 by Miles, Misra and Irwin who at the time were working at the LSHTM. The Miles and Misra method has been shown to be precise.

Materials

- A calibrated dropping pipette, delivering drops of 20-200µl.
- Petri dishes containing nutrient agar.
- Phosphate Buffered Saline (PBS)
- Bacterial suspension or homogenate.

Method

- The inoculum / suspension is serially diluted by adding 20µl of suspension to 180µl of PBS. When the quantity of bacteria is unknown, dilutions are made to at least 10^6.
- The plates are divided into six equal sectors.
- Onto each section, 60 µl (3 x 20 µl) of the appropriate dilution is plated.
- The plates are incubated for 18 – 24 hours and observed for growth.
- Colonies are counted in the sector where 30-300 colonies are visible.
- The following equation is used to calculate the number of colony forming units (CFU) per ml from the original aliquot/ sample:

  CFU per ml = Number of colonies in sector x dilution factor x (1000/60).

Streaking

A plate which has been streaked showing the colonies thinning as the streaking moves clockwise.

In microbiology, **Streaking** is a technique used to isolate a pure strain from a single species of microorganism, often bacteria. Samples can then be taken from the resulting colonies and a microbiological culture can be grown on a new plate so that the organism can be identified, studied, or tested.

The streaking is done using a sterile tool, such as a cotton swab or commonly an inoculation loop. This is dipped in an inoculum such as a broth or patient specimen containing many species of bacteria.

The sample is spread across one quadrant of a petri dish containing a growth medium, usually an agar plate which has been sterilized in an autoclave. Choice of which growth medium is used depends on which microorganism is being cultured, or selected for. Growth media are usually forms of agar, a gelatinous substance derived from seaweed.

Different labs have different standards as to the direction and style of the streaking.

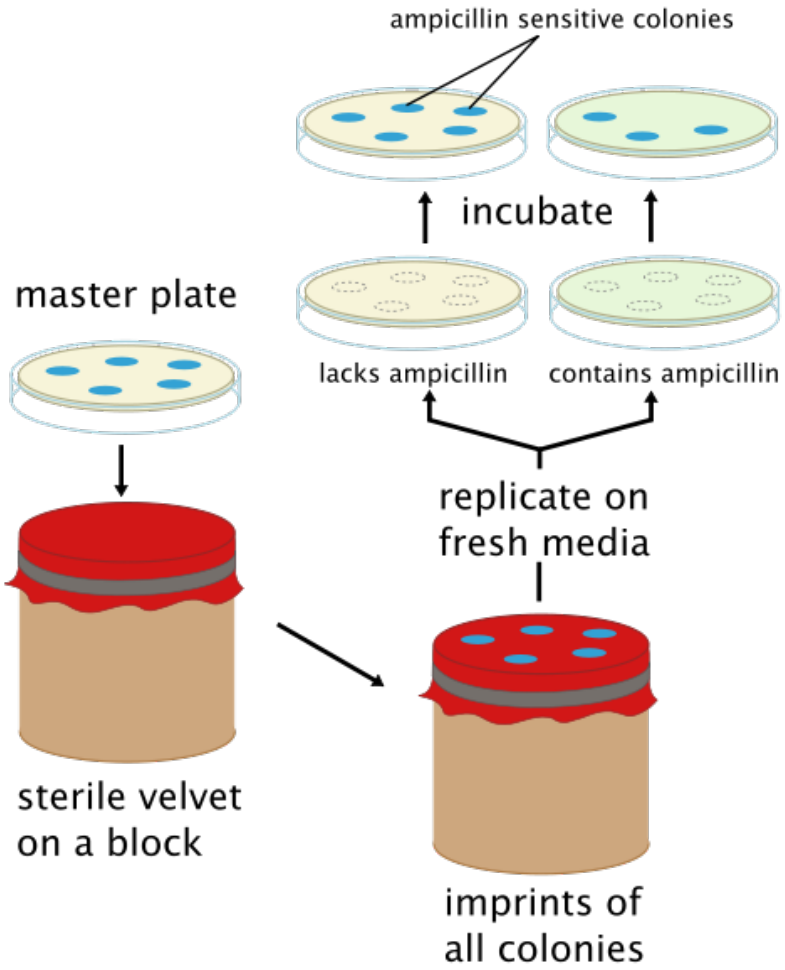
The quadrant which is first inoculated will contain too many bacteria to select one colony. By re-sterilising the loop and dragged it across the previously inoculated quadrant, only some of the original sample is introduced to new sections of the plate. The loop is re-sterilised and a new quadrant inoculated in the same manner. Each time the loop gathers fewer and fewer bacteria until it gathers just single bacterial cells that can grow into a colony.

Dependant on the strain, the plate may then be incubated, usually for 24 to 36 hours, to allow the bacteria to reproduce. At the end of incubation there should be enough bacteria to form visible colonies in the areas touched by the inoculation loop. From these mixed

colonies, single bacterial or fungal species can be identified based on their morphological (size/shape/colour) differences, and then sub-cultured to a new media plate to yield a pure culture for further analysis.

Automated equipments are used at industrial level for streak plating the solid media in order to achieve better sterilization and consistency of streaking and for reliably faster work. While streaking manually it is important to avoid scratching the solid medium as subsequent streak lines will be damaged and non-uniform deposition of inoculum at damaged sites on the medium yield clustered growth of microbes which may extend into nearby streak lines.

Replica plating



Negative selection through replica plating to screen for ampicillin sensitive colonies

In molecular biology and microbiology, **replica plating** is a technique in which one or more secondary Petri plates containing different solid (agar-based) selective growth media (lacking nutrients or containing chemical growth inhibitors such as antibiotics) are inoculated with the same colonies of microorganisms from a primary plate (or master dish), reproducing the original spatial pattern of colonies. The technique involves pressing a velvet-covered disk, nitrocellulose membrane, or filter paper to a primary plate, and then imprinting secondary plates with cells in colonies removed from the original plate by the material. Generally, large numbers of colonies (roughly 30-300) are replica plated due to the difficulty in streaking each out individually onto a separate plate.

The purpose of replica plating is to be able to compare the master plate and any secondary plates to screen for a selectable phenotype. For example, a colony which appeared on the master plate but failed to appear at the same location on a secondary plate shows that the colony was sensitive to a substance on that particular secondary plate. Common screenable phenotypes include auxotrophy and antibiotic resistance.

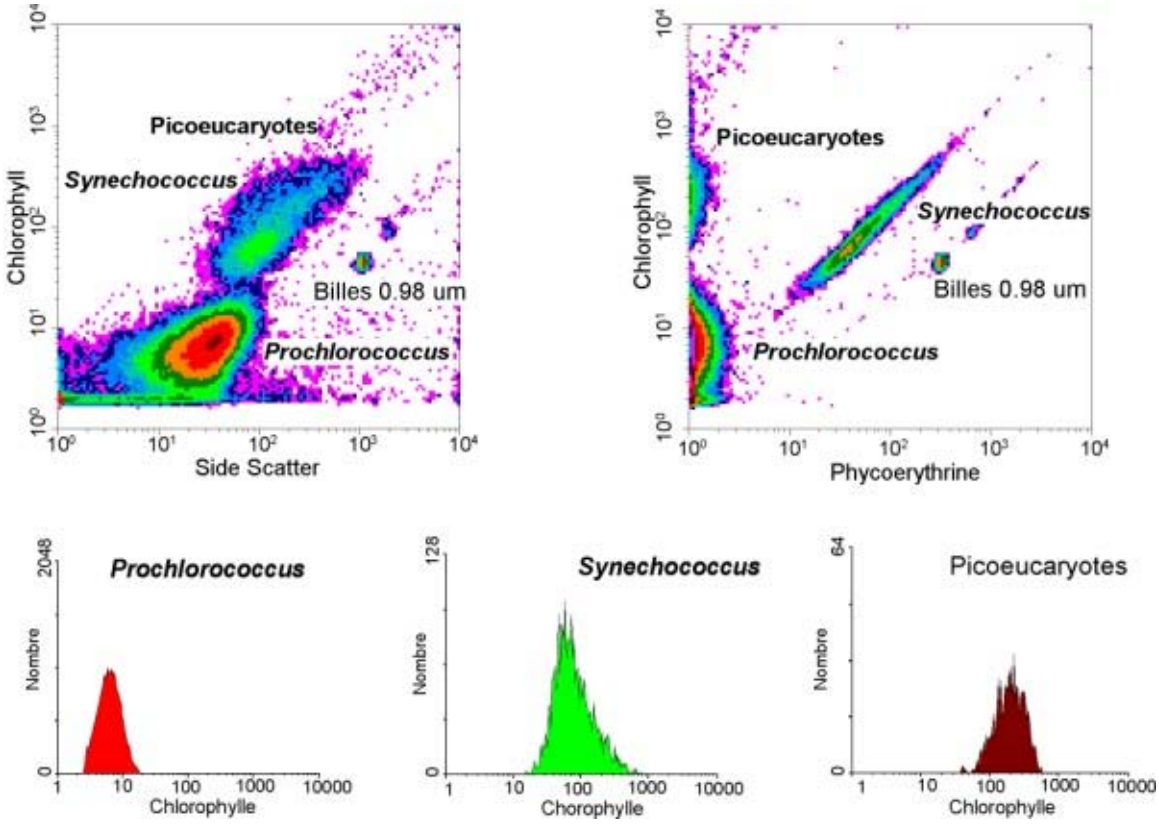
Replica plating is especially useful for "negative selection". However, it's more correct to refer to "negative screening" instead of using the term 'selection'. For example, if one wanted to select colonies that were sensitive to ampicillin, the primary plate could be replica plated on a secondary Amp^+ agar plate . The sensitive colonies on the secondary plate would die but the colonies could still be deduced from the primary plate since the two have the same spatial patterns from ampicillin resistant colonies. The sensitive colonies could then be picked off from the primary plate. While doing this, frequently the last plate will be non-selective, in this example, a nonselective plate will be replica plated after the Amp+ plate, to confirm that the absence of growth on the selective plate is due to the selection itself, and not a problem with transferring cells. Basically, if one sees growth on the third (nonselective) plate but not the second one, this indicates the selective agent is responsible for the lack of growth; if the non-selective plate shows no growth then one cannot say whether viable cells were transferred at all and no conclusions can be made about the presence or absence of growth on selective media. This is particularly useful if there are questions about the age or viability of the cells on the original plate.

By increasing the variety of secondary plates with different selective growth media, it is possible to rapidly screen a large number of individual isolated colonies for as many phenotypes as there are secondary plates.

This technique was first described by Joshua Lederberg and Esther Lederberg in 1952.

Chapter- 11

Flow Cytometry



Analysis of a marine sample of photosynthetic picoplankton by flow cytometry showing three different populations (*Prochlorococcus*, *Synechococcus*, and picoeukaryotes)

Flow cytometry (abbreviated: **FCM**) is a technique for counting and examining microscopic particles, such as cells and chromosomes, by suspending them in a stream of fluid and passing them by an electronic detection apparatus. It allows simultaneous multiparametric analysis of the physical and/or chemical characteristics of up to thousands of particles per second. Flow cytometry is routinely used in the diagnosis of health disorders, especially blood cancers, but has many other applications in both

research and clinical practice. A common variation is to physically sort particles based on their properties, so as to purify populations of interest.

History

The first impedance-based flow cytometry device, using the Coulter principle, was disclosed in U.S. Patent 2,656,508, issued in 1953, to Wallace H. Coulter. The first fluorescence-based flow cytometry device (ICP 11) was developed in 1968 by Wolfgang Göhde from the University of Münster and first commercialized in 1968/69 by German developer and manufacturer Partec through Phywe AG in Göttingen. At that time, absorption methods were still widely favored by other scientists over fluorescence methods . Soon after, flow cytometry instruments were developed, including the Cytofluorograph (1971) from Bio/Physics Systems Inc. (later: Ortho Diagnostics), the PAS 8000 (1973) from Partec, the first FACS instrument from Becton Dickinson (1974), the ICP 22 (1975) from Partec/Phywe and the Epics from Coulter (1977/78).

Name of the technology

The original name of the flow cytometry technology was "pulse cytophotometry" (German: *Impulszytophotometrie*). Only 20 years later in 1988, at the Conference of the American Engineering Foundation in Pensacola, Florida, the name was changed to "flow cytometry", a term that quickly became popular.

Principle of flow cytometry

A beam of light (usually laser light) of a **single wavelength** is directed onto a hydrodynamically-focused stream of fluid. A number of detectors are aimed at the point where the stream passes through the light beam: one in line with the light beam (Forward Scatter or FSC) and several perpendicular to it (Side Scatter (SSC) and one or more fluorescent detectors). Each suspended particle from 0.2 to 150 micrometers passing through the beam scatters the ray, and fluorescent chemicals found in the particle or attached to the particle may be excited into emitting light at a longer wavelength than the light source. This combination of scattered and fluorescent light is picked up by the detectors, and, by analysing fluctuations in brightness at each detector (one for each fluorescent emission peak), it is then possible to derive various types of information about the physical and chemical structure of each individual particle. FSC correlates with the cell volume and SSC depends on the inner complexity of the particle (i.e., shape of the nucleus, the amount and type of cytoplasmic granules or the membrane roughness). Some flow cytometers on the market have eliminated the need for fluorescence and use only light scatter for measurement. Other flow cytometers form images of each cell's fluorescence, scattered light, and transmitted light.

Front of desktop flow cytometer - the Becton-Dickinson FACSCalibur.

Flow cytometers

Modern flow cytometers are able to analyze several thousand particles every second, in "real time," and can actively separate and isolate particles having specified properties. A flow cytometer is similar to a microscope, except that, instead of producing an image of the cell, flow cytometry offers "high-throughput" (for a large number of cells) automated quantification of set parameters. To analyze solid tissues, a single-cell suspension must first be prepared.

A flow cytometer has 5 main components:

- a flow cell - liquid stream (sheath fluid), which carries and aligns the cells so that they pass single file through the light beam for sensing
- a measuring system - commonly used are measurement of impedance (or conductivity) and optical systems - lamps (mercury, xenon); high-power water-cooled lasers (argon, krypton, dye laser); low-power air-cooled lasers (argon (488 nm), red-HeNe (633 nm), green-HeNe, HeCd (UV)); diode lasers (blue, green, red, violet) resulting in light signals

- a detector and Analogue-to-Digital Conversion (ADC) system - which generates FSC and SSC as well as fluorescence signals from light into electrical signals that can be processed by a computer
- an amplification system - linear or logarithmic
- a computer for analysis of the signals.

The process of collecting data from samples using the flow cytometer is termed 'Acquisition'. Acquisition is mediated by a computer physically connected to the flow cytometer, and the software which handles the digital interface with the cytometer. The software is capable of adjusting parameters (i.e. voltage, compensation, etc.) for the sample being tested, and also assists in displaying initial sample information while acquiring sample data to insure that parameters are set correctly. Early flow cytometers were, in general, experimental devices, but technological advances have enabled widespread applications for use in a variety of both clinical and research purposes. Due to these developments, a considerable market for instrumentation, analysis software, as well as the reagents used in acquisition such as fluorescently-labeled antibodies has developed.

Modern instruments usually have multiple lasers and fluorescence detectors (the current record for a commercial instrument is **4 lasers and 18 fluorescence detectors**). Increasing the number of lasers and detectors allows for multiple antibody labeling, and can more precisely identify a target population by their phenotypic markers. Certain instruments can even take digital images of individual cells, allowing for the analysis of fluorescent signal location within or on the surface of cell.

Data analysis

Gating

The data generated by flow-cytometers can be plotted in a single dimension, to produce a histogram, or in two-dimensional dot plots or even in three dimensions. The regions on these plots can be sequentially separated, based on fluorescence intensity, by creating a series of subset extractions, termed "gates." Specific gating protocols exist for diagnostic and clinical purposes especially in relation to hematology.

The plots are often made on logarithmic scales. Because different fluorescent dyes' emission spectra overlap, signals at the detectors have to be compensated electronically as well as computationally. Data accumulated using the flow cytometer can be analyzed using software, e.g., WinMDI(deprecated), Flowjo, FCS Express, VenturiOne or CellQuest Pro. Once the data is collected, there is no need to stay connected to the flow cytometer. For this reason, analysis is most often done on a separate computer. This is especially necessary in core facilities where usage of these machines is in high demand.

Computational analysis

Recent progress on automated population identification using computational methods has offered an alternative to traditional gating strategies. Automated identification systems could potentially help findings of rare and hidden populations. Representative automated methods include FLOCK in Immunology Database and Analysis Portal (ImmPort) , FLAME in GenePattern and flowClust ,, in Bioconductor. Collaborative efforts have resulted in an open project called FlowCAP (Flow Cytometry: Critical Assessment of Population Identification Methods,) to provide an objective way to compare and evaluate the flow cytometry data clustering methods, and also to establish guidance about appropriate use and application of these methods.

Fluorescence-activated cell sorting

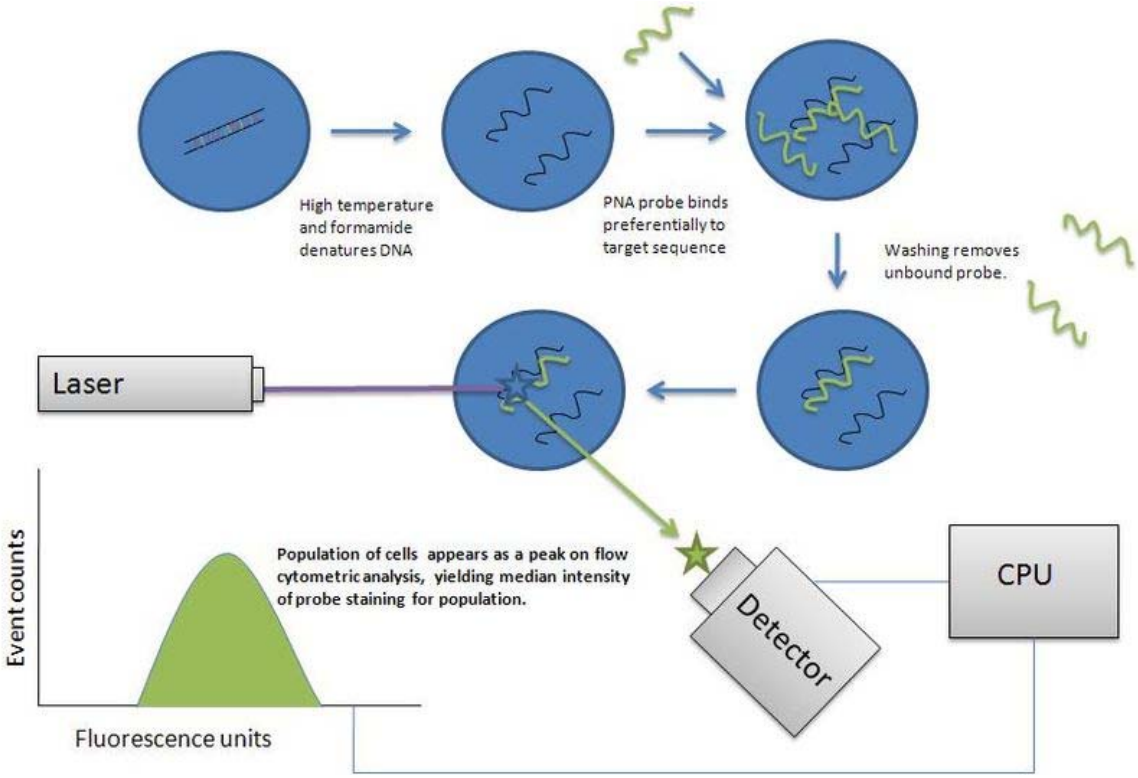
Fluorescence-activated cell sorting is a specialized type of flow cytometry. It provides a method for sorting a heterogeneous mixture of biological cells into two or more containers, one cell at a time, based upon the specific light scattering and fluorescent characteristics of each cell. It is a useful scientific instrument, as it provides fast, objective and quantitative recording of fluorescent signals from individual cells as well as physical separation of cells of particular interest. The acronym FACS is trademarked and owned by Becton, Dickinson and Company. While many immunologists use this term frequently for all types of sorting and non-sorting applications, it is not a generic term for flow cytometry. The first cell sorter was invented by Mack Fulwyler in 1965, using the Coulter principle, a relatively difficult technique and one no longer used in modern instruments. The technique was expanded by Len Herzenberg who was responsible for coining the term FACS. Herzenberg won the Kyoto Prize in 2006 for his work in flow cytometry.

The cell suspension is entrained in the center of a narrow, rapidly flowing stream of liquid. The flow is arranged so that there is a large separation between cells relative to their diameter. A vibrating mechanism causes the stream of cells to break into individual droplets. The system is adjusted so that there is a low probability of more than one cell per droplet. Just before the stream breaks into droplets, the flow passes through a fluorescence measuring station where the fluorescent character of interest of each cell is measured. An electrical charging ring is placed just at the point where the stream breaks into droplets. A charge is placed on the ring based on the immediately-prior fluorescence intensity measurement, and the opposite charge is trapped on the droplet as it breaks from the stream. The charged droplets then fall through an electrostatic deflection system that diverts droplets into containers based upon their charge. In some systems, the charge is applied directly to the stream, and the droplet breaking off retains charge of the same sign as the stream. The stream is then returned to neutral after the droplet breaks off.

Fluorescent labels

A wide range of fluorophores can be used as labels in flow cytometry. These each have a characteristic peak excitation and emission wavelength. Also, the emission spectra of the

labels often overlap. Consequently, the combination of labels which can be used depends on the wavelength of the lamp(s) or laser(s) used to excite the fluorochromes and on the detectors available



Use of flow cytometry to measure copy number variation of a specific DNA sequence (Flow-FISH)

Measurable parameters

This list is very long and constantly expanding.

- volume and morphological complexity of cells
- cell pigments such as chlorophyll or phycoerythrin
- total DNA content (cell cycle analysis, cell kinetics, proliferation, etc.)
- total RNA content
- DNA copy number variation (by Flow-FISH)
- chromosome analysis and sorting (library construction, chromosome paint)
- protein expression and localization
- Protein modifications, phospho-proteins
- transgenic products *in vivo*, particularly the Green fluorescent protein or related fluorescent * cell surface antigens (Cluster of differentiation (CD) markers)
- intracellular antigens (various cytokines, secondary mediators, etc.)
- nuclear antigens

- enzymatic activity
- pH, intracellular ionized calcium, magnesium, membrane potential
- membrane fluidity
- apoptosis (quantification, measurement of DNA degradation, mitochondrial membrane potential, permeability changes, caspase activity)
- cell viability
- monitoring electropermeabilization of cells
- oxidative burst
- characterising multidrug resistance (MDR) in cancer cells
- glutathione
- various combinations (DNA/surface antigens, etc.)
- cell adherence (for instance pathogen-host cell adherence)

Applications

The technology has applications in a number of fields, including molecular biology, pathology, immunology, plant biology and marine biology. It has broad application in medicine (especially in transplantation, hematology, tumor immunology and chemotherapy, genetics and sperm sorting for sex preselection). In marine biology, the auto-fluorescent properties of photosynthetic plankton can be exploited by flow cytometry in order to characterise abundance and community structure. In protein engineering, flow cytometry is used in conjunction with yeast display and bacterial display to identify cell surface-displayed protein variants with desired properties. It is also used to determine ploidy of grass carp fry.