



# Cell Signaling and Imaging

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## Chapter 1

# Cell Signaling

**Cell signaling** is part of a complex system of communication that governs basic cellular activities and coordinates cell actions. The ability of cells to perceive and correctly respond to their microenvironment is the basis of development, tissue repair, and immunity as well as normal tissue homeostasis. Errors in cellular information processing are responsible for diseases such as cancer, autoimmunity, and diabetes. By understanding cell signaling, diseases may be treated effectively and, theoretically, artificial tissues may be created.

Traditional work in biology has focused on studying individual parts of cell signaling pathways. Systems biology research helps us to understand the underlying structure of cell signaling networks and how changes in these networks may affect the transmission and flow of information. Such networks are complex systems in their organization and may exhibit a number of emergent properties including bistability and ultrasensitivity. Analysis of cell signaling networks requires a combination of experimental and theoretical approaches including the development and analysis of simulations and modelling.

## ***Unicellular and multicellular organism cell signaling***

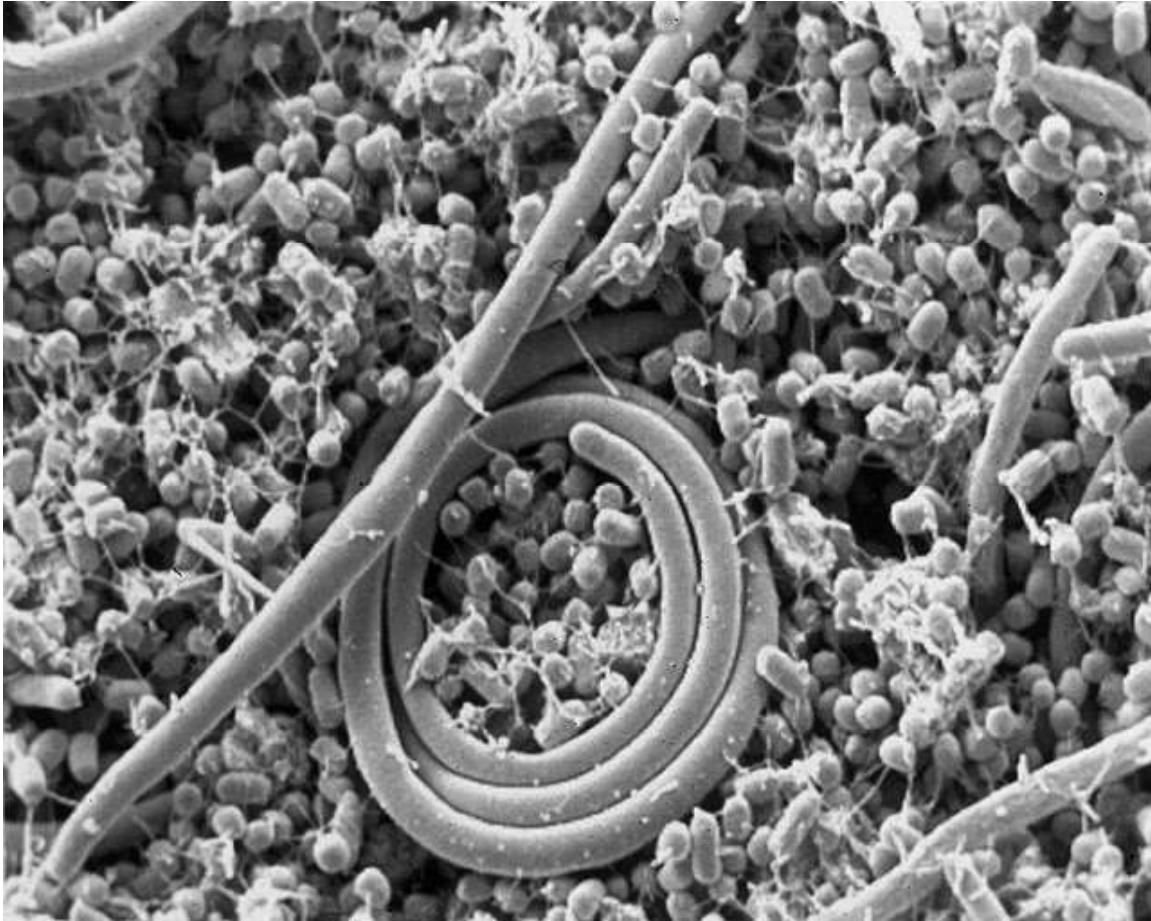


Figure 1. Example of signaling between bacteria. *Salmonella enteritidis* uses acyl-homoserine lactone for Quorum sensing (see: Inter-Bacterial Communication)

Cell signaling has been most extensively studied in the context of human diseases and signaling between cells of a single organism. However, cell signaling may also occur between the cells of two different organisms. In many mammals, early embryo cells exchange signals with cells of the uterus. In the human gastrointestinal tract, bacteria exchange signals with each other and with human epithelial and immune system cells. For the yeast *Saccharomyces cerevisiae* during mating, some cells send a peptide signal (mating factor *pheromones*) into their environment. The mating factor peptide may bind to a cell surface receptor on other yeast cells and induce them to prepare for mating.

## Types of signals

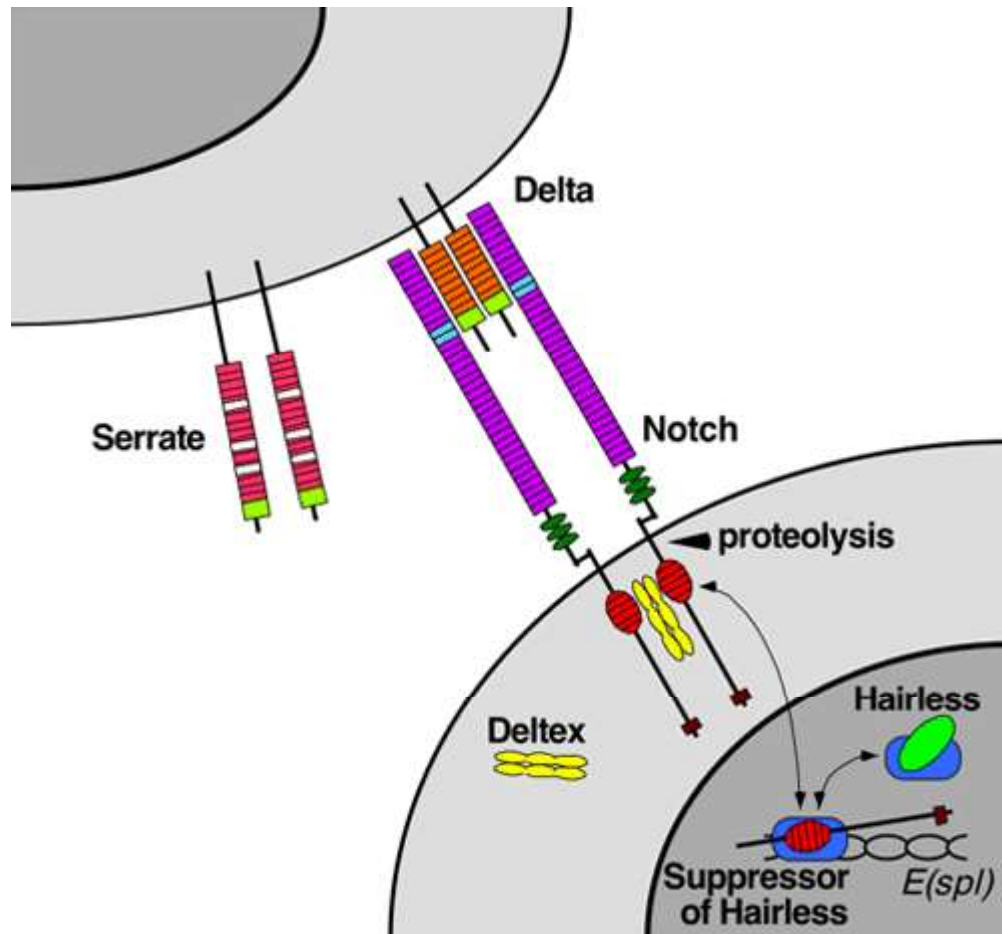


Figure 2. Notch-mediated juxtacrine signal between adjacent cells.

Cells communicate with each other via direct contact (juxtacrine signaling), over short distances (paracrine signaling), or over large distances and/or scales (endocrine signaling).

Some cell-to-cell communication requires direct cell-cell contact. Some cells can form gap junctions that connect their cytoplasm to the cytoplasm of adjacent cells. In cardiac muscle, gap junctions between adjacent cells allows for action potential propagation from the cardiac pacemaker region of the heart to spread and coordinately cause contraction of the heart.

The Notch signaling mechanism is an example of juxtacrine signalling (also known as contact-dependent signaling) in which two adjacent cells must make physical contact in order to communicate. This requirement for direct contact allows for very precise control of cell differentiation during embryonic development. In the worm *Caenorhabditis elegans*, two cells of the developing gonad each have an equal chance of terminally differentiating or becoming a uterine precursor cell that continues to divide. The choice of which cell continues to divide is controlled by competition of cell surface signals. One

cell will happen to produce more of a cell surface protein that activates the Notch receptor on the adjacent cell. This activates a feedback loop or system that reduces Notch expression in the cell that will differentiate and that increases Notch on the surface of the cell that continues as a stem cell.

Many cell signals are carried by molecules that are released by one cell and move to make contact with another cell. *Endocrine* signals are called hormones. Hormones are produced by endocrine cells and they travel through the blood to reach all parts of the body. Specificity of signaling can be controlled if only some cells can respond to a particular hormone. *Paracrine* signals such as retinoic acid target only cells in the vicinity of the emitting cell. Neurotransmitters represent another example of a paracrine signal. Some signaling molecules can function as both a hormone and a neurotransmitter. For example, epinephrine and norepinephrine can function as hormones when released from the adrenal gland and are transported to the heart by way of the blood stream. Norepinephrine can also be produced by neurons to function as a neurotransmitter within the brain. Estrogen can be released by the ovary and function as a hormone or act locally via paracrine or autocrine signaling. Active species of oxygen and nitric oxide can also act as cellular messengers. This process is dubbed redox signaling.

## **Receptors for cell moves**

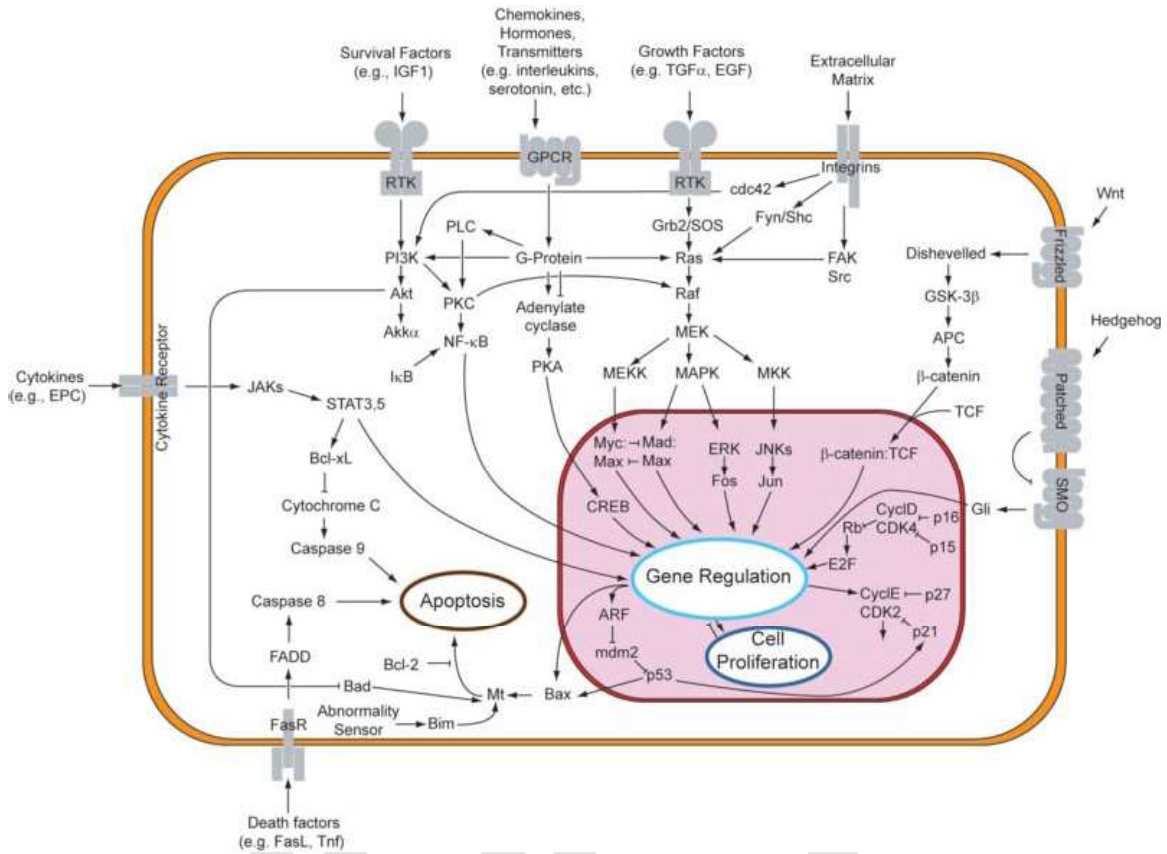
Cells receive information from their environment through a class of proteins known as receptors. Notch is a cell surface protein that functions as a receptor. Animals have a small set of genes that code for signaling proteins that interact specifically with Notch receptors and stimulate a response in cells that express Notch on their surface. Molecules that activate (or, in some cases, inhibit) receptors can be classified as hormones, neurotransmitters, cytokines, growth factors but all of these are called receptor ligands. The details of ligand-receptor interactions are fundamental to cell signaling.

As shown in Figure 2 (above, left), Notch acts as a receptor for ligands that are expressed on adjacent cells. While many receptors are cell surface proteins, some are found inside cells. For example, oestrogen is a hydrophobic molecule that can pass through the lipid bilayer of cell surface membranes. Oestrogen receptors inside cells of the uterus can be activated by oestrogen that comes from the ovaries, enters the target cells, and binds to oestrogen receptors.

A number of transmembrane receptors for molecules that include peptide hormones and of intracellular receptors for steroid hormones exist, giving to a cell the ability to respond to a great number of hormonal and pharmacological stimuli. In diseases, often, proteins that interact with receptors are aberrantly activated, resulting in constitutively activated downstream signals.

For several types of intercellular signaling molecules that are unable to permeate the hydrophobic cell membrane due to their hydrophilic nature, the target receptor is expressed on the membrane. When such signaling molecule activates its receptor, the signal is carried into the cell usually by means of a second messenger such as cAMP.

# Signaling pathways



Overview of signal transduction pathways.

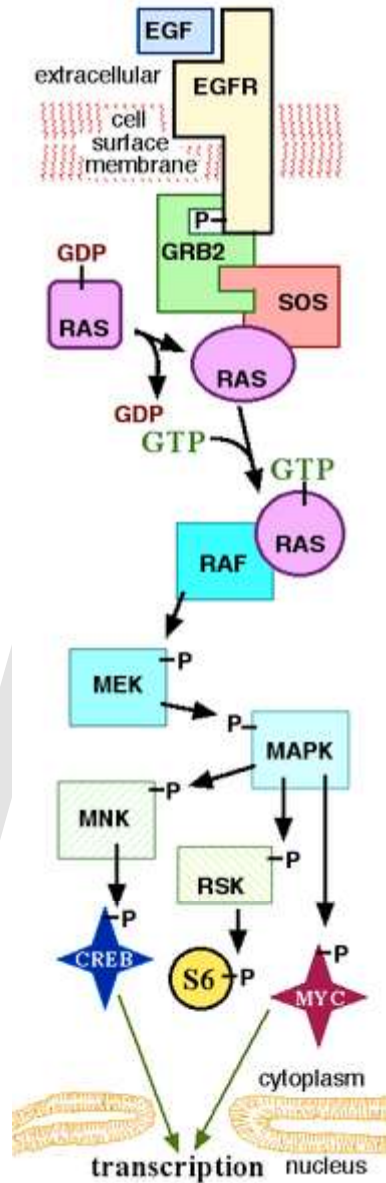


Figure 3. Diagram showing key components of a signal transduction pathway.

In some cases, receptor activation caused by ligand binding to a receptor is directly coupled to the cell's response to the ligand. For example, the neurotransmitter GABA can activate a cell surface receptor that is part of an ion channel. GABA binding to a GABA A receptor on a neuron opens a chloride-selective ion channel that is part of the receptor. GABA A receptor activation allows negatively-charged chloride ions to move into the neuron, which inhibits the ability of the neuron to produce action potentials. However, for many cell surface receptors, ligand-receptor interactions are not directly linked to the cell's response. The activated receptor must first interact with other proteins inside the cell before the ultimate physiological effect of the ligand on the cell's behavior is produced. Often, the behavior of a chain of several interacting cell proteins is altered

following receptor activation. The entire set of cell changes induced by receptor activation is called a signal transduction mechanism or pathway.

In the case of Notch-mediated signaling, the signal transduction mechanism can be relatively simple. As shown in Figure 2 (above, left), activation of Notch can cause the Notch protein to be altered by a protease. Part of the Notch protein is released from the cell surface membrane and can act to change the pattern of gene transcription in the cell nucleus. This causes the responding cell to make different proteins, resulting in an altered pattern of cell behavior. Cell signaling research involves studying the spatial and temporal dynamics of both receptors and the components of signaling pathways that are activated by receptors in various cell types.

A more complex signal transduction pathway is shown in Figure 3. This pathway involves changes of protein-protein interactions inside the cell, induced by an external signal. Many growth factors bind to receptors at the cell surface and stimulate cells to progress through the cell cycle and divide. Several of these receptors are kinases that start to phosphorylate themselves and other proteins when binding to a ligand. This phosphorylation can generate a binding site for a different protein and thus induce protein-protein interaction. In Figure 3, the ligand (called epidermal growth factor (EGF)) binds to the receptor (called EGFR). This activates the receptor to phosphorylate itself. The phosphorylated receptor binds to an adaptor protein (GRB2), which couples the signal to further downstream signaling processes. For example, one of the signal transduction pathways that are activated is called the mitogen-activated protein kinase (MAPK) pathway. The signal transduction component labeled as "MAPK" in the pathway was originally called "ERK," so the pathway is called the MAPK/ERK pathway. The MAPK protein is an enzyme, a protein kinase that can attach phosphate to target proteins such as the transcription factor MYC and, thus, alter gene transcription and, ultimately, cell cycle progression. Many cellular proteins are activated downstream of the growth factor receptors (such as EGFR) that initiate this signal transduction pathway.

Some signaling transduction pathways respond differently depending on the amount of signaling received by the cell. For instance, the hedgehog protein activates different genes, depending on the amount of hedgehog protein present.

Complex multi-component signal transduction pathways provide opportunities for feedback, signal amplification, and interactions inside one cell between multiple signals and signaling pathways.

### ***Classification of intercellular communication***

Within endocrinology (the study of intercellular signalling in animals) and the endocrine system, intercellular signalling is subdivided into the following classifications:

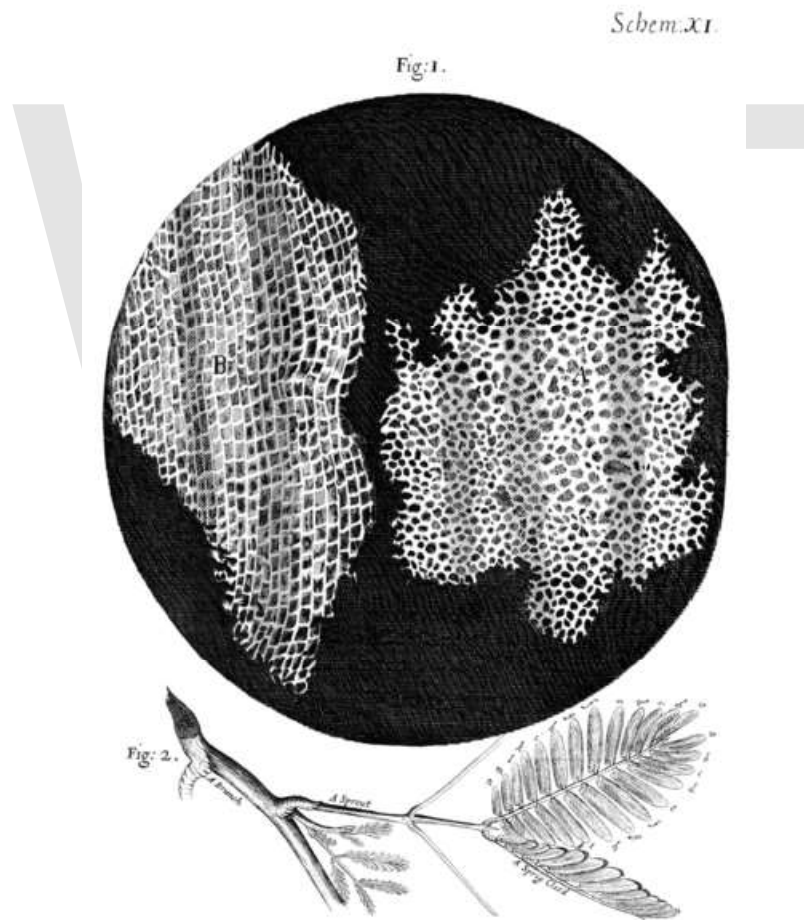
- *Intracrine* signals are produced within the target cell.

- *Autocrine* signals target the cell itself. Sometimes autocrine cells can target cells close by if they are the same type of cell as the emitting cell. An example of this are immune cells.
- *Juxtacrine* signals target adjacent (touching) cells. These signals are transmitted along cell membranes via protein or lipid components integral to the membrane and are capable of affecting either the emitting cell or cells immediately adjacent.
- *Paracrine* signals target cells in the vicinity of the emitting cell. Neurotransmitters represent an example.
- *Endocrine* signals target distant cells. Endocrine cells produce hormones that travel through the blood to reach all parts of the body.

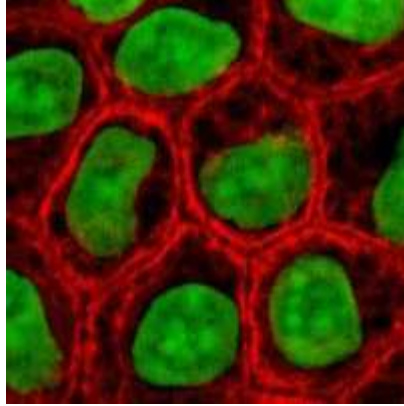
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## Chapter 2

# Cell (Biology)



Drawing of the structure of cork as it appeared under the microscope to Robert Hooke from *Micrographia* which is the origin of the word "cell" being used to describe the smallest unit of a living organism



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

The **cell** is the functional basic unit of life. It was discovered by Robert Hooke and is the functional unit of all known living organisms. It is the smallest unit of life that is classified as a living thing, and is often called the building block of life. Some organisms, such as most bacteria, are unicellular (consist of a single cell). Other organisms, such as humans, are multicellular. Humans have about 100 trillion or  $10^{14}$  cells; a typical cell size is  $10\ \mu\text{m}$  and a typical cell mass is 1 nanogram. The largest cells are about  $135\ \mu\text{m}$  in the anterior horn in the spinal cord while granule cells in the cerebellum, the smallest, can be some  $4\ \mu\text{m}$  and the longest cell can reach from the toe to the lower brain stem (Pseudounipolar cells). The largest known cells are unfertilised ostrich egg cells which weigh 3.3 pounds.

In 1835, before the final cell theory was developed, Jan Evangelista Purkyně observed small "granules" while looking at the plant tissue through a microscope. The cell theory, first developed in 1839 by Matthias Jakob Schleiden and Theodor Schwann, states that all organisms are composed of one or more cells, that all cells come from preexisting cells, that vital functions of an organism occur within cells, and that all cells contain the hereditary information necessary for regulating cell functions and for transmitting information to the next generation of cells.

The word *cell* comes from the Latin *cellula*, meaning, a small room. The descriptive term for the smallest living biological structure was coined by Robert Hooke in a book he published in 1665 when he compared the cork cells he saw through his microscope to the small rooms monks lived in.

## **Anatomy**

There are two types of cells: eukaryotic and prokaryotic. Prokaryotic cells are usually independent, while eukaryotic cells are often found in multicellular organisms.

## Prokaryotic cells

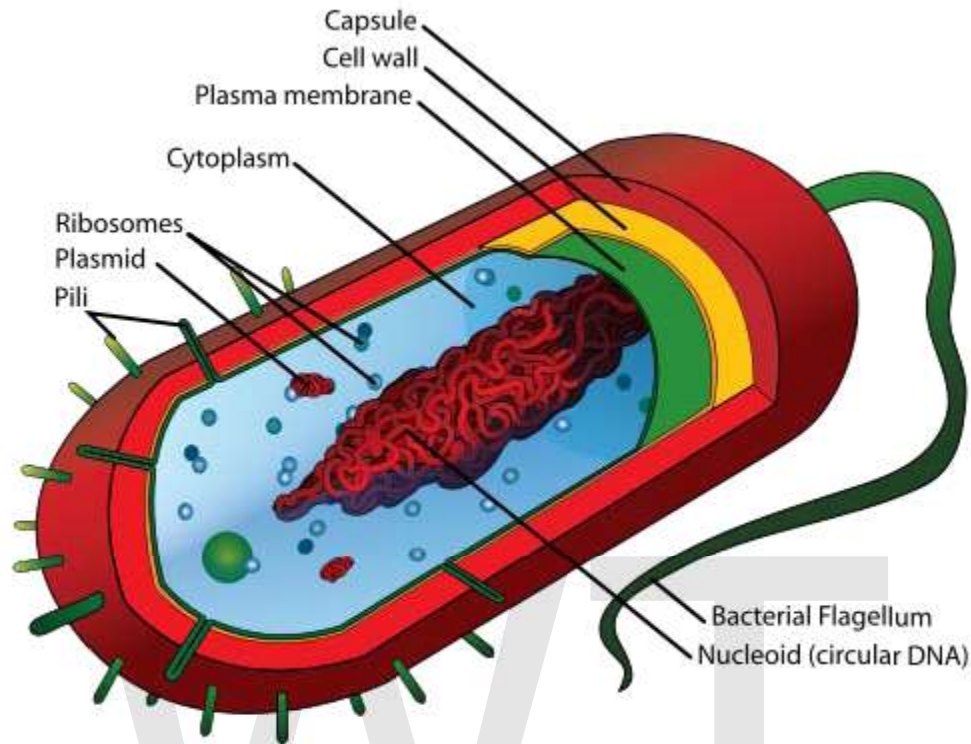


Diagram of a typical prokaryotic cell

The prokaryote cell is simpler, and therefore smaller, than a eukaryote cell, lacking a nucleus and most of the other organelles of eukaryotes. There are two kinds of prokaryotes: bacteria and archaea; these share a similar structure.

Nuclear material of prokaryotic cell consist of a single chromosome which is in direct contact with cytoplasm. Here the undefined nuclear region in the cytoplasm is called nucleoid.

A prokaryotic cell has three architectural regions:

- On the outside, flagella and pili project from the cell's surface. These are structures (not present in all prokaryotes) made of proteins that facilitate movement and communication between cells;
- Enclosing the cell is the cell envelope – generally consisting of a cell wall covering a plasma membrane though some bacteria also have a further covering layer called a capsule. The envelope gives rigidity to the cell and separates the interior of the cell from its environment, serving as a protective filter. Though most prokaryotes have a cell wall, there are exceptions such as *Mycoplasma* (bacteria) and *Thermoplasma* (archaea). The cell wall consists of *peptidoglycan* in bacteria, and acts as an additional barrier against exterior forces. It also prevents

the cell from expanding and finally bursting (cytolysis) from osmotic pressure against a hypotonic environment. Some eukaryote cells (plant cells and fungi cells) also have a cell wall;

- Inside the cell is the cytoplasmic region that contains the cell genome (DNA) and ribosomes and various sorts of inclusions. A prokaryotic chromosome is usually a circular molecule (an exception is that of the bacterium *Borrelia burgdorferi*, which causes Lyme disease). Though not forming a *nucleus*, the DNA is condensed in a *nucleoid*. Prokaryotes can carry extrachromosomal DNA elements called *plasmids*, which are usually circular. Plasmids enable additional functions, such as antibiotic resistance.

## Eukaryotic cells

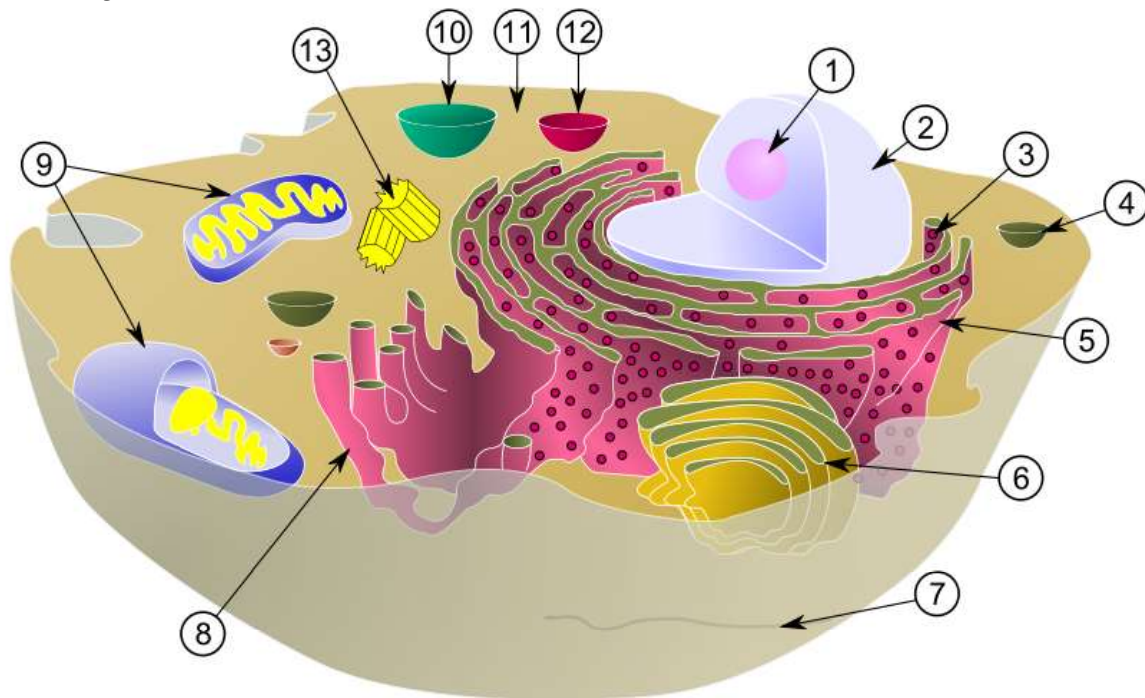


Diagram of a typical animal (eukaryotic) cell, showing subcellular components.

Organelles:

- (1) nucleolus
- (2) nucleus
- (3) ribosome
- (4) vesicle
- (5) rough endoplasmic reticulum (ER)
- (6) Golgi apparatus
- (7) Cytoskeleton
- (8) smooth endoplasmic reticulum
- (9) mitochondria
- (10) vacuole
- (11) cytoplasm

- (12) lysosome
- (13) centrioles within centrosome

Eukaryotic cells are about 15 times wider than a typical prokaryote and can be as much as 1000 times greater in volume. The major difference between prokaryotes and eukaryotes is that eukaryotic cells contain membrane-bound compartments in which specific metabolic activities take place. Most important among these is a cell nucleus, a membrane-delineated compartment that houses the eukaryotic cell's DNA. This nucleus gives the eukaryote its name, which means "true nucleus." Other differences include:

- The plasma membrane resembles that of prokaryotes in function, with minor differences in the setup. Cell walls may or may not be present.
- The eukaryotic DNA is organized in one or more linear molecules, called chromosomes, which are associated with histone proteins. All chromosomal DNA is stored in the *cell nucleus*, separated from the cytoplasm by a membrane. Some eukaryotic organelles such as mitochondria also contain some DNA.
- Many eukaryotic cells are ciliated with *primary cilia*. Primary cilia play important roles in chemosensation, mechanosensation, and thermosensation. Cilia may thus be "viewed as sensory cellular antennae that coordinate a large number of cellular signaling pathways, sometimes coupling the signaling to ciliary motility or alternatively to cell division and differentiation."
- Eukaryotes can move using *motile cilia* or *flagella*. The flagella are more complex than those of prokaryotes.

**Table 1: Comparison of features of prokaryotic and eukaryotic cells**

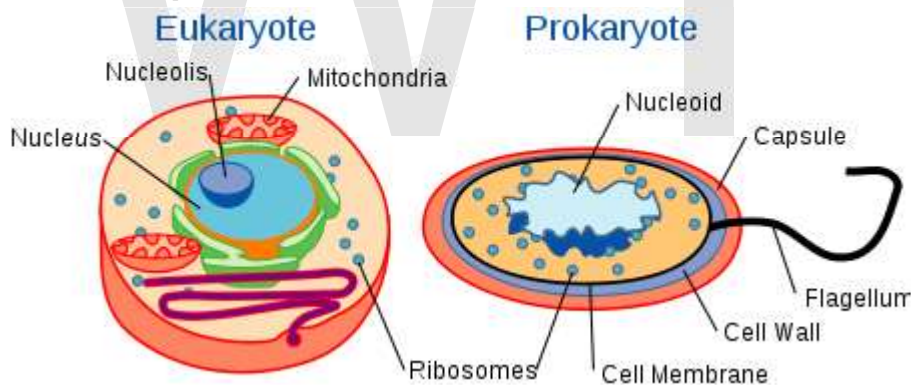
	<b>Prokaryotes</b>	<b>Eukaryotes</b>
<b>Typical organisms</b>	bacteria, archaea	protists, fungi, plants, animals
<b>Typical size</b>	~ 1–10 μm	~ 10–100 μm (sperm cells, apart from the tail, are smaller)
<b>Type of nucleus</b>	nucleoid region; no real nucleus	real nucleus with double membrane
<b>DNA</b>	circular (usually)	linear molecules (chromosomes) with histone proteins
<b>RNA-/protein-synthesis</b>	coupled in cytoplasm	RNA-synthesis inside the nucleus protein synthesis in cytoplasm
<b>Ribosomes</b>	50S+30S	60S+40S
<b>Cytoplasmatic structure</b>	very few structures	highly structured by endomembranes and a cytoskeleton
<b>Cell movement</b>	flagella made of flagellin	flagella and cilia containing microtubules; lamellipodia and filopodia containing actin
<b>Mitochondria</b>	none	one to several thousand (though some lack mitochondria)
<b>Chloroplasts</b>	none	in algae and plants
<b>Organization</b>	usually single cells	single cells, colonies, higher multicellular

		organisms with specialized cells
<b>Cell division</b>	Binary fission (simple division)	Mitosis (fission or budding) Meiosis

**Table 2: Comparison of structures between animal and plant cells**

	<b>Typical animal cell</b>	<b>Typical plant cell</b>
<b>Organelles</b>	<ul style="list-style-type: none"> <li>• Nucleus               <ul style="list-style-type: none"> <li>◦ Nucleolus (within nucleus)</li> </ul> </li> <li>• Rough endoplasmic reticulum (ER)</li> <li>• Smooth ER</li> <li>• Ribosomes</li> <li>• Cytoskeleton</li> <li>• Golgi apparatus</li> <li>• Cytoplasm</li> <li>• Mitochondria</li> <li>• Vesicles</li> <li>• Lysosomes</li> <li>• Centrosome               <ul style="list-style-type: none"> <li>◦ Centrioles</li> </ul> </li> </ul>	<ul style="list-style-type: none"> <li>• Nucleus               <ul style="list-style-type: none"> <li>◦ Nucleolus (within nucleus)</li> </ul> </li> <li>• Rough ER</li> <li>• Smooth ER</li> <li>• Ribosomes</li> <li>• Cytoskeleton</li> <li>• Golgi apparatus (dictyosomes)</li> <li>• Cytoplasm</li> <li>• Mitochondria</li> <li>• Plastids and its derivatives</li> <li>• Vacuole(s)</li> <li>• Cell wall</li> </ul>

### **Subcellular components**



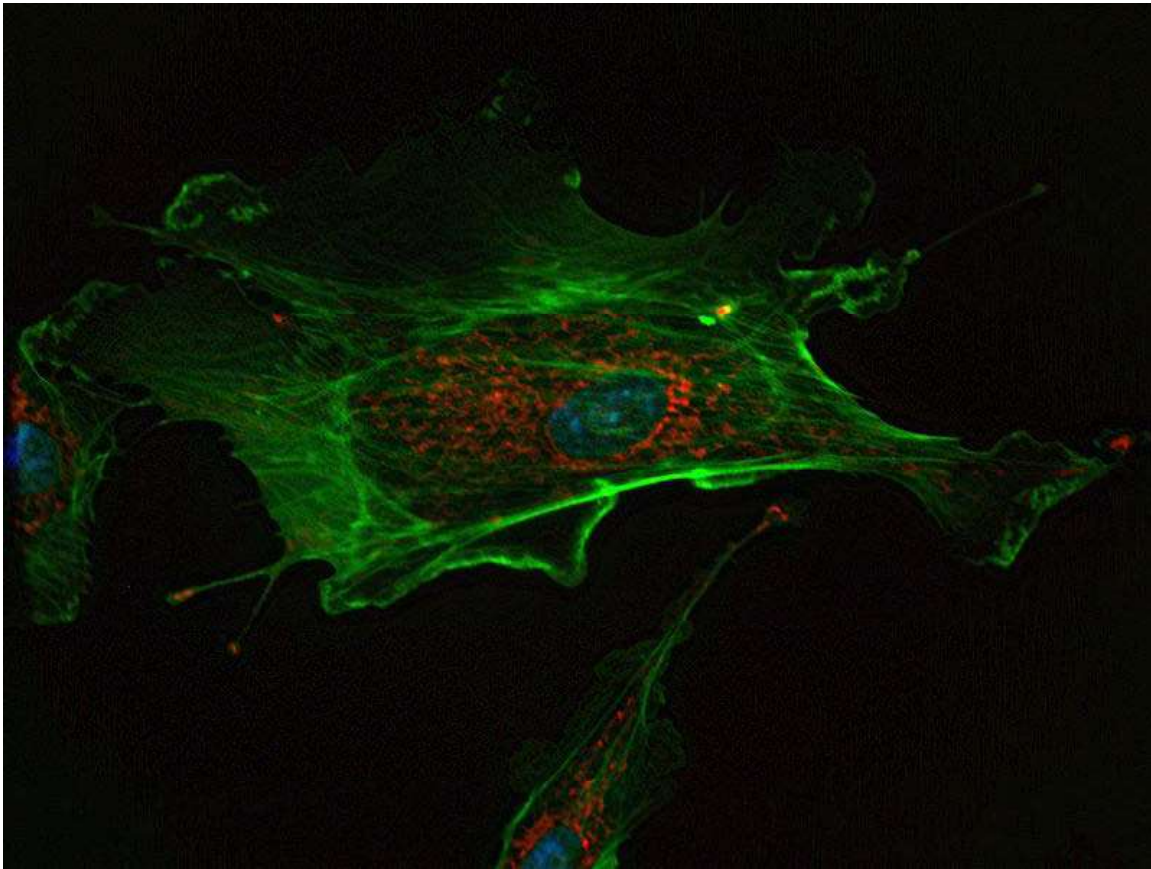
The cells of eukaryotes (left) and prokaryotes (right)

All cells, whether prokaryotic or eukaryotic, have a membrane that envelops the cell, separates its interior from its environment, regulates what moves in and out (selectively permeable), and maintains the electric potential of the cell. Inside the membrane, a salty cytoplasm takes up most of the cell volume. All cells possess DNA, the hereditary material of genes, and RNA, containing the information necessary to build various proteins such as enzymes, the cell's primary machinery. There are also other kinds of biomolecules in cells.

## Membrane

The cytoplasm of a cell is surrounded by a cell membrane or *plasma membrane*. The plasma membrane in plants and prokaryotes is usually covered by a cell wall. This membrane serves to separate and protect a cell from its surrounding environment and is made mostly from a double layer of lipids (hydrophobic fat-like molecules) and hydrophilic phosphorus molecules. Hence, the layer is called a phospholipid bilayer. It may also be called a fluid mosaic membrane. Embedded within this membrane is a variety of protein molecules that act as channels and pumps that move different molecules into and out of the cell. The membrane is said to be 'semi-permeable', in that it can either let a substance (molecule or ion) pass through freely, pass through to a limited extent or not pass through at all. Cell surface membranes also contain receptor proteins that allow cells to detect external signaling molecules such as hormones.

## Cytoskeleton



Bovine Pulmonary Artery Endothelial cell: nuclei stained blue, mitochondria stained red, and F-actin, an important component in microfilaments, stained green. Cell imaged on a fluorescent microscope.

The cytoskeleton acts to organize and maintain the cell's shape; anchors organelles in place; helps during endocytosis, the uptake of external materials by a cell, and cytokinesis, the separation of daughter cells after cell division; and moves parts of the

cell in processes of growth and mobility. The eukaryotic cytoskeleton is composed of microfilaments, intermediate filaments and microtubules. There is a great number of proteins associated with them, each controlling a cell's structure by directing, bundling, and aligning filaments. The prokaryotic cytoskeleton is less well-studied but is involved in the maintenance of cell shape, polarity and cytokinesis.

## **Genetic material**

Two different kinds of genetic material exist: deoxyribonucleic acid (DNA) and ribonucleic acid (RNA). Most organisms use DNA for their long-term information storage, but some viruses (e.g., retroviruses) have RNA as their genetic material. The biological information contained in an organism is encoded in its DNA or RNA sequence. RNA is also used for information transport (e.g., mRNA) and enzymatic functions (e.g., ribosomal RNA) in organisms that use DNA for the genetic code itself. Transfer RNA (tRNA) molecules are used to add amino acids during protein translation.

Prokaryotic genetic material is organized in a simple circular DNA molecule (the bacterial chromosome) in the nucleoid region of the cytoplasm. Eukaryotic genetic material is divided into different, linear molecules called chromosomes inside a discrete nucleus, usually with additional genetic material in some organelles like mitochondria and chloroplasts.

A human cell has genetic material contained in the cell nucleus (the nuclear genome) and in the mitochondria (the mitochondrial genome). In humans the nuclear genome is divided into 23 pairs of linear DNA molecules called chromosomes. The mitochondrial genome is a circular DNA molecule distinct from the nuclear DNA. Although the mitochondrial DNA is very small compared to nuclear chromosomes, it codes for 13 proteins involved in mitochondrial energy production and specific tRNAs.

Foreign genetic material (most commonly DNA) can also be artificially introduced into the cell by a process called transfection. This can be transient, if the DNA is not inserted into the cell's genome, or stable, if it is. Certain viruses also insert their genetic material into the genome.

## **Organelles**

The human body contains many different organs, such as the heart, lung, and kidney, with each organ performing a different function. Cells also have a set of "little organs," called organelles, that are adapted and/or specialized for carrying out one or more vital functions. Both eukaryotic and prokaryotic cells have organelles but organelles in eukaryotes are generally more complex and may be membrane bound.

There are several types of organelles in a cell. Some (such as the nucleus and golgi apparatus) are typically solitary, while others (such as mitochondria, peroxisomes and lysosomes) can be numerous (hundreds to thousands). The cytosol is the gelatinous fluid that fills the cell and surrounds the organelles.

Cell nucleus – eukaryotes only - a cell's information center

The cell nucleus is the most conspicuous organelle found in a eukaryotic cell. It houses the cell's chromosomes, and is the place where almost all DNA replication and RNA synthesis (transcription) occur. The nucleus is spherical and separated from the cytoplasm by a double membrane called the nuclear envelope. The nuclear envelope isolates and protects a cell's DNA from various molecules that could accidentally damage its structure or interfere with its processing. During processing, DNA is transcribed, or copied into a special RNA, called messenger RNA (mRNA). This mRNA is then transported out of the nucleus, where it is translated into a specific protein molecule. The nucleolus is a specialized region within the nucleus where ribosome subunits are assembled. In prokaryotes, DNA processing takes place in the cytoplasm.



Diagram of a cell nucleus

Mitochondria and Chloroplasts – eukaryotes only - the power generators

Mitochondria are self-replicating organelles that occur in various numbers, shapes, and sizes in the cytoplasm of all eukaryotic cells. Mitochondria play a critical role in generating energy in the eukaryotic cell. Mitochondria generate the cell's energy by oxidative phosphorylation, using oxygen to release energy stored in cellular nutrients (typically pertaining to glucose) to generate ATP. Mitochondria multiply by splitting in two. Respiration occurs in the cell mitochondria. Organelles that are modified chloroplasts are broadly called plastids, and are involved in energy storage through photosynthesis, which uses solar energy to generate carbohydrates and oxygen from carbon dioxide and water. Mitochondria and chloroplasts each contain their own genome, which is separate and distinct from the nuclear genome of a cell. Both organelles contain this DNA in circular plasmids, much like prokaryotic cells, strongly supporting the evolutionary theory of endosymbiosis; since these organelles contain

their own genomes and have other similarities to prokaryotes, they are thought to have developed through a symbiotic relationship after being engulfed by a primitive cell.

#### Endoplasmic reticulum – eukaryotes only

The endoplasmic reticulum (ER) is the transport network for molecules targeted for certain modifications and specific destinations, as compared to molecules that will float freely in the cytoplasm. The ER has two forms: the rough ER, which has ribosomes on its surface and secretes proteins into the cytoplasm, and the smooth ER, which lacks them. Smooth ER plays a role in calcium sequestration and release.

#### Golgi apparatus – eukaryotes only

The primary function of the Golgi apparatus is to process and package the macromolecules such as proteins and lipids that are synthesized by the cell. It is particularly important in the processing of proteins for secretion. The Golgi apparatus forms a part of the endomembrane system of eukaryotic cells. Vesicles that enter the Golgi apparatus are processed in a cis to trans direction, meaning they coalesce on the cis side of the apparatus and after processing pinch off on the opposite (trans) side to form a new vesicle in the animal cell.



Diagram of an endomembrane system

#### Ribosomes

The ribosome is a large complex of RNA and protein molecules. They each consist of two subunits, and act as an assembly line where RNA from the nucleus is used to synthesise proteins from amino acids. Ribosomes can be found either floating freely or bound to a membrane (the rough endoplasmic reticulum in eukaryotes, or the cell membrane in prokaryotes).

#### Lysosomes and Peroxisomes – eukaryotes only

Lysosomes contain digestive enzymes (acid hydrolases). They digest excess or worn-out organelles, food particles, and engulfed viruses or bacteria. Peroxisomes have enzymes that rid the cell of toxic peroxides. The cell could not house these

destructive enzymes if they were not contained in a membrane-bound system. These organelles are often called a "suicide bag" because of their ability to detonate and destroy the cell.

**Centrosome** – the cytoskeleton organiser

The centrosome produces the microtubules of a cell – a key component of the cytoskeleton. It directs the transport through the ER and the Golgi apparatus. Centrosomes are composed of two centrioles, which separate during cell division and help in the formation of the mitotic spindle. A single centrosome is present in the animal cells. They are also found in some fungi and algae cells.

**Vacuoles**

Vacuoles store food and waste. Some vacuoles store extra water. They are often described as liquid filled space and are surrounded by a membrane. Some cells, most notably *Amoeba*, have contractile vacuoles, which can pump water out of the cell if there is too much water. The vacuoles of eukaryotic cells are usually larger in those of plants than animals.

## **Structures outside the cell wall**

### **Capsule**

A gelatinous capsule is present in some bacteria outside the cell wall. The capsule may be polysaccharide as in pneumococci, meningococci or polypeptide as *Bacillus anthracis* or hyaluronic acid as in streptococci. Capsules are not marked by ordinary stain and can be detected by special stain. The capsule is antigenic. The capsule has antiphagocytic function so it determines the virulence of many bacteria. It also plays a role in attachment of the organism to mucous membranes.

### **Flagella**

Flagella are the organelles of cellular mobility. They arise from cytoplasm and extrude through the cell wall. They are long and thick thread-like appendages, protein in nature. Are most commonly found in bacteria cells but are found in animal cells as well.

### **Fimbriae (pili)**

They are short and thin hair like filaments, formed of protein called pilin (antigenic). Fimbriae are responsible for attachment of bacteria to specific receptors of human cell (adherence). There are special types of pili called (sex pili) involved in conjugation.

## **Functions**

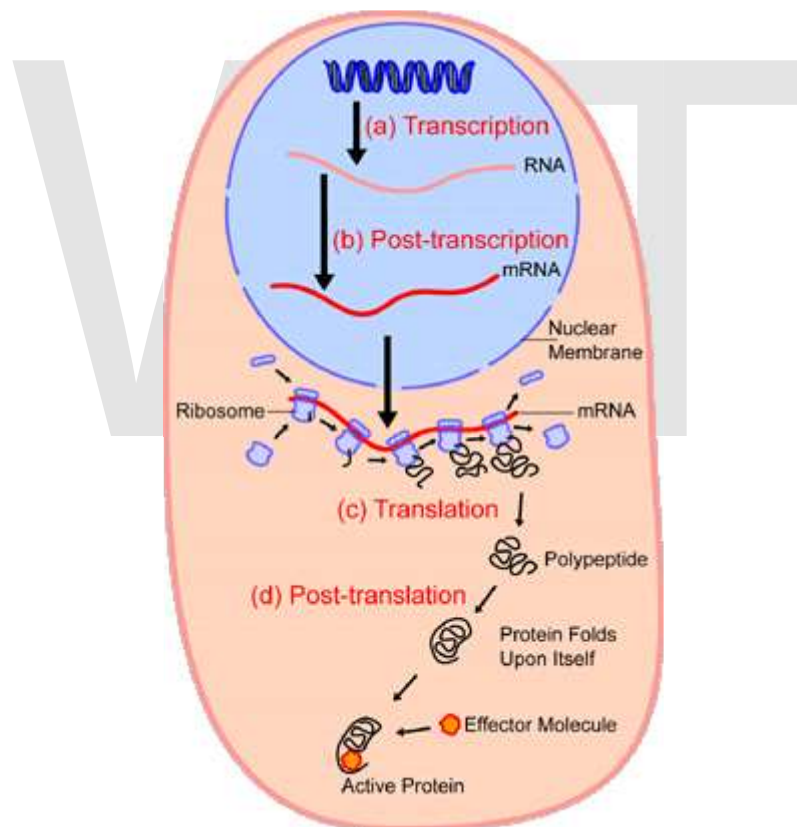
### **Growth and metabolism**

Between successive cell divisions, cells grow through the functioning of cellular metabolism. Cell metabolism is the process by which individual cells process nutrient

molecules. Metabolism has two distinct divisions: catabolism, in which the cell breaks down complex molecules to produce energy and reducing power, and anabolism, in which the cell uses energy and reducing power to construct complex molecules and perform other biological functions. Complex sugars consumed by the organism can be broken down into a less chemically complex sugar molecule called glucose. Once inside the cell, glucose is broken down to make adenosine triphosphate (ATP), a form of energy, through two different pathways.

The first pathway, glycolysis, requires no oxygen and is referred to as anaerobic metabolism. Each reaction is designed to produce some hydrogen ions that can then be used to make energy packets (ATP). In prokaryotes, glycolysis is the only method used for converting energy.

The second pathway, called the Krebs cycle, or citric acid cycle, occurs inside the mitochondria and can generate enough ATP to run all the cell functions.



An overview of protein synthesis.

Within the nucleus of the cell (*light blue*), genes (DNA, *dark blue*) are transcribed into RNA. This RNA is then subject to post-transcriptional modification and control, resulting in a mature mRNA (*red*) that is then transported out of the nucleus and into the cytoplasm (*peach*), where it undergoes translation into a protein. mRNA is translated by ribosomes (*purple*) that match the three-base codons of the mRNA to the three-base anti-codons of the appropriate tRNA. Newly synthesized proteins (*black*) are often further modified, such as by binding to an effector molecule (*orange*), to become fully active.

## **Creation**

Cell division involves a single cell (called a *mother cell*) dividing into two daughter cells. This leads to growth in multicellular organisms (the growth of tissue) and to procreation (vegetative reproduction) in unicellular organisms.

Prokaryotic cells divide by binary fission. Eukaryotic cells usually undergo a process of nuclear division, called mitosis, followed by division of the cell, called cytokinesis. A diploid cell may also undergo meiosis to produce haploid cells, usually four. Haploid cells serve as gametes in multicellular organisms, fusing to form new diploid cells.

DNA replication, or the process of duplicating a cell's genome, is required every time a cell divides. Replication, like all cellular activities, requires specialized proteins for carrying out the job.

## **Protein synthesis**

Cells are capable of synthesizing new proteins, which are essential for the modulation and maintenance of cellular activities. This process involves the formation of new protein molecules from amino acid building blocks based on information encoded in DNA/RNA. Protein synthesis generally consists of two major steps: transcription and translation.

Transcription is the process where genetic information in DNA is used to produce a complementary RNA strand. This RNA strand is then processed to give messenger RNA (mRNA), which is free to migrate through the cell. mRNA molecules bind to protein-RNA complexes called ribosomes located in the cytosol, where they are translated into polypeptide sequences. The ribosome mediates the formation of a polypeptide sequence based on the mRNA sequence. The mRNA sequence directly relates to the polypeptide sequence by binding to transfer RNA (tRNA) adapter molecules in binding pockets within the ribosome. The new polypeptide then folds into a functional three-dimensional protein molecule.

## ***Movement or motility***

Cells can move during many processes: such as wound healing, the immune response and cancer metastasis. For wound healing to occur, white blood cells and cells that ingest bacteria move to the wound site to kill the microorganisms that cause infection.

At the same time fibroblasts (connective tissue cells) move there to remodel damaged structures. In the case of tumor development, cells from a primary tumor move away and spread to other parts of the body. Cell motility involves many receptors, crosslinking, bundling, binding, adhesion, motor and other proteins. The process is divided into three steps – protrusion of the leading edge of the cell, adhesion of the leading edge and de-adhesion at the cell body and rear, and cytoskeletal contraction to pull the cell forward. Each step is driven by physical forces generated by unique segments of the cytoskeleton.

## ***Evolution***

The origin of cells has to do with the origin of life, which began the history of life on Earth.

### **Origin of the first cell**

There are several theories about the origin of small molecules that could lead to life in an early Earth. One is that they came from meteorites. Another is that they were created at deep-sea vents. A third is that they were synthesized by lightning in a reducing atmosphere; although it is not clear if Earth had such an atmosphere. There are essentially no experimental data defining what the first self-replicating forms were. RNA is generally assumed to be the earliest self-replicating molecule, as it is capable of both storing genetic information and catalyzing chemical reactions. But some other entity with the potential to self-replicate could have preceded RNA, like clay or peptide nucleic acid.

Cells emerged at least 4.0–4.3 billion years ago. The current belief is that these cells were heterotrophs. An important characteristic of cells is the cell membrane, composed of a bilayer of lipids. The early cell membranes were probably more simple and permeable than modern ones, with only a single fatty acid chain per lipid. Lipids are known to spontaneously form bilayered vesicles in water, and could have preceded RNA. But the first cell membranes could also have been produced by catalytic RNA, or even have required structural proteins before they could form.

### **Origin of eukaryotic cells**

The eukaryotic cell seems to have evolved from a symbiotic community of prokaryotic cells. DNA-bearing organelles like the mitochondria and the chloroplasts are almost certainly what remains of ancient symbiotic oxygen-breathing proteobacteria and cyanobacteria, respectively, where the rest of the cell seems to be derived from an ancestral archaean prokaryote cell – a theory termed the endosymbiotic theory.

There is still considerable debate about whether organelles like the hydrogenosome predated the origin of mitochondria, or viceversa.

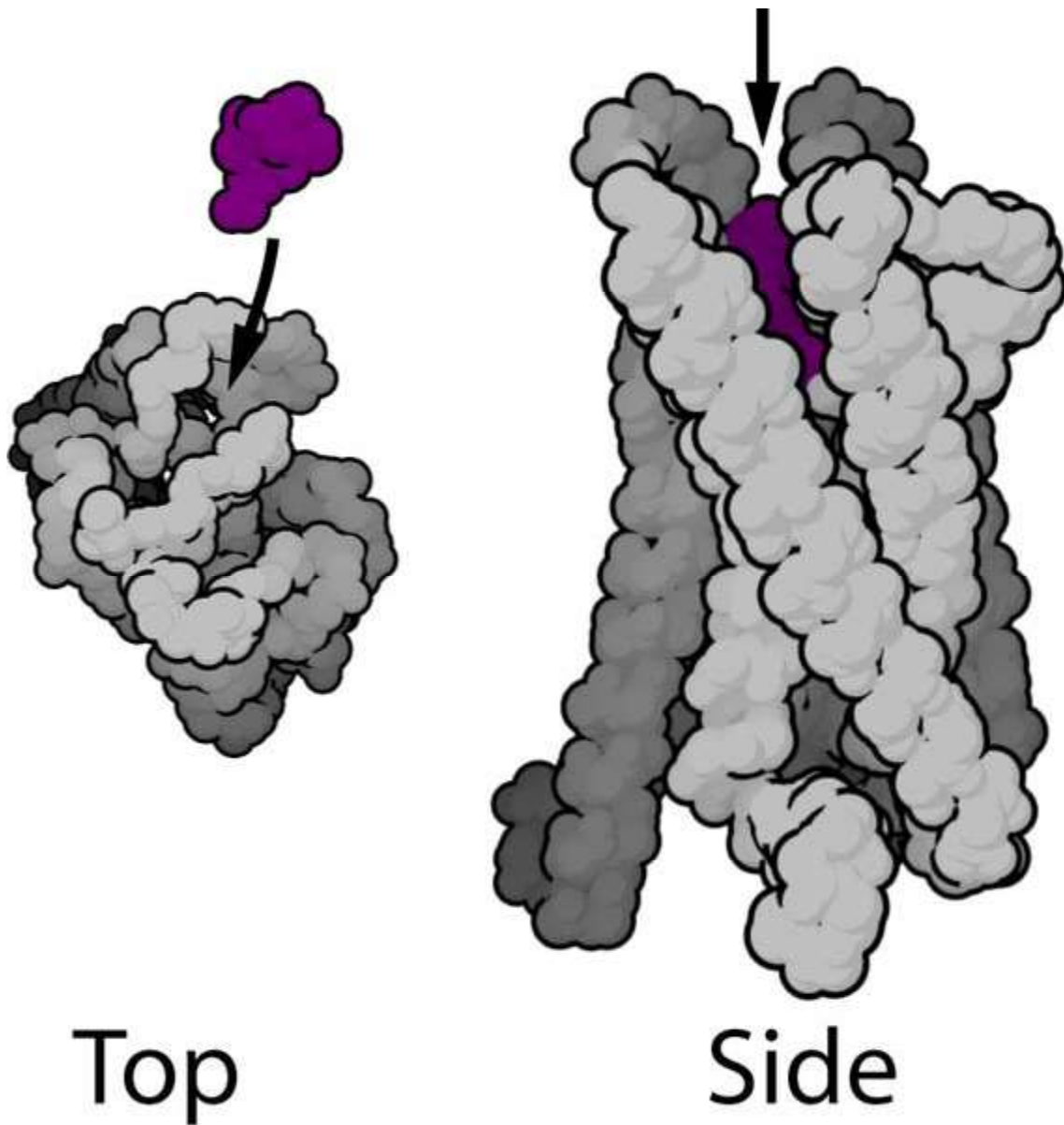
Sex, as the stereotyped choreography of meiosis and syngamy that persists in nearly all extant eukaryotes, may have played a role in the transition from prokaryotes to eukaryotes. An 'origin of sex as vaccination' theory suggests that the eukaryote genome accreted from prokaryotic parasite genomes in numerous rounds of lateral gene transfer. Sex-as-syngamy (fusion sex) arose when infected hosts began swapping nuclearized genomes containing co-evolved, vertically transmitted symbionts that conveyed protection against horizontal infection by more virulent symbionts.

## History

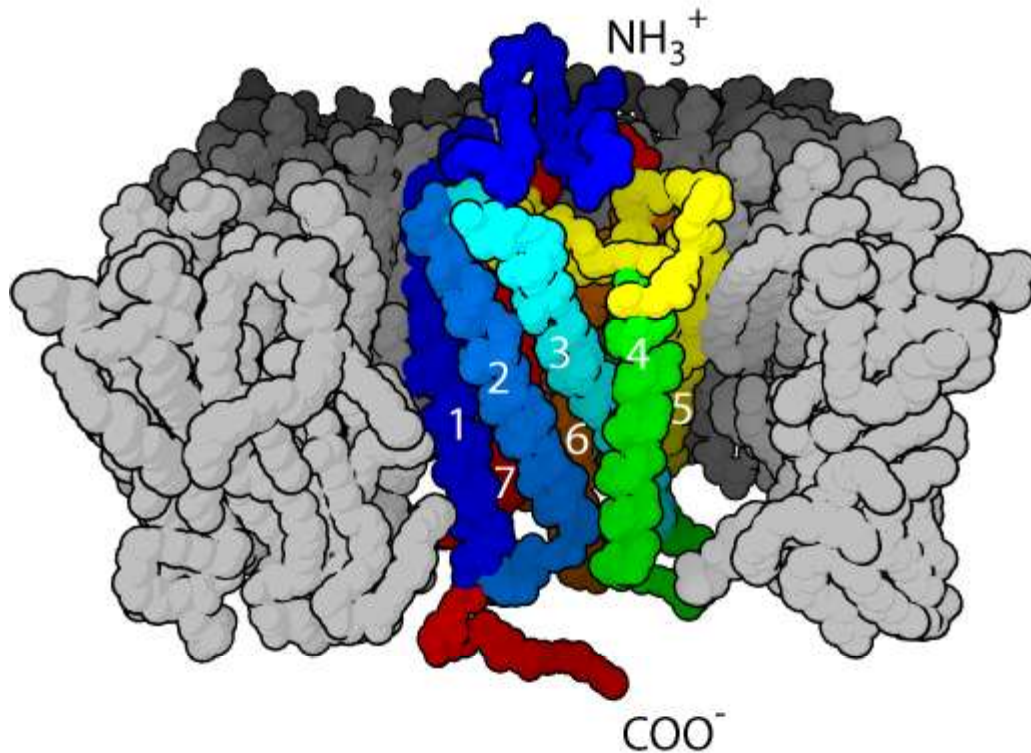
- 1632–1723: Antonie van Leeuwenhoek teaches himself to grind lenses, builds a microscope and draws protozoa, such as *Vorticella* from rain water, and bacteria from his own mouth.
- 1665: Robert Hooke discovers cells in cork, then in living plant tissue using an early microscope.
- 1839: Theodor Schwann and Matthias Jakob Schleiden elucidate the principle that plants and animals are made of cells, concluding that cells are a common unit of structure and development, and thus founding the cell theory.
- The belief that life forms can occur spontaneously (*generatio spontanea*) is contradicted by Louis Pasteur (1822–1895) (although Francesco Redi had performed an experiment in 1668 that suggested the same conclusion).
- 1855: Rudolf Virchow states that cells always emerge from cell divisions (*omnis cellula ex cellula*).
- 1931: Ernst Ruska builds first transmission electron microscope (TEM) at the University of Berlin. By 1935, he has built an EM with twice the resolution of a light microscope, revealing previously unresolvable organelles.
- 1953: Watson and Crick made their first announcement on the double-helix structure for DNA on February 28.
- 1981: Lynn Margulis published *Symbiosis in Cell Evolution* detailing the endosymbiotic theory.

## Chapter 3

# G protein-Coupled Receptor



A mu-opioid G-protein-coupled receptor with its agonist

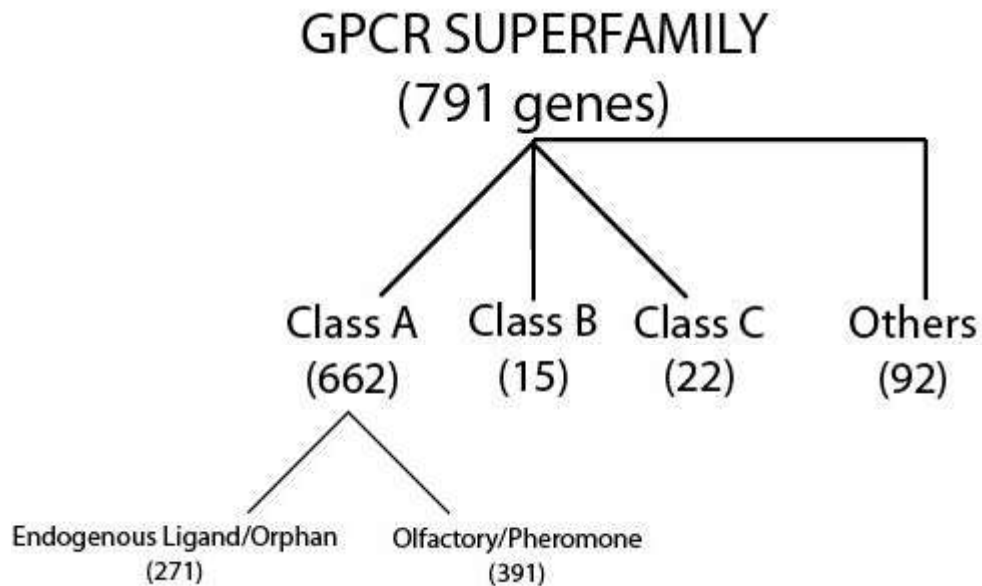


The seven-transmembrane  $\alpha$ -helix structure of a G-protein-coupled receptor

**G protein-coupled receptors (GPCRs)**, also known as **seven-transmembrane domain receptors**, **7TM receptors**, **heptahelical receptors**, **serpentine receptor**, and **G protein-linked receptors (GPLR)**, comprise a large protein family of transmembrane receptors that sense molecules outside the cell and activate inside signal transduction pathways and, ultimately, cellular responses. G protein-coupled receptors are found only in eukaryotes, including yeast, choanoflagellates, and animals. The ligands that bind and activate these receptors include light-sensitive compounds, odors, pheromones, hormones, and neurotransmitters, and vary in size from small molecules to peptides to large proteins. G protein-coupled receptors are involved in many diseases, and are also the target of approximately 30% of all modern medicinal drugs.

There are two principal signal transduction pathways involving the G protein-coupled receptors: the cAMP signal pathway and the Phosphatidylinositol signal pathway. When a ligand binds to the GPCR it causes a conformational change in the GPCR, which allows it to act as a guanine nucleotide exchange factor (GEF). The GPCR can then activate an associated G-protein by exchanging its bound GDP for a GTP. The G-protein's  $\alpha$  subunit, together with the bound GTP, can then dissociate from the  $\beta$  and  $\gamma$  subunits to further affect intracellular signaling proteins or target functional proteins directly depending on the  $\alpha$  subunit type ( $G_{\alpha s}$ ,  $G_{\alpha i/o}$ ,  $G_{\alpha q/11}$ ,  $G_{\alpha 12/13}$ ).

## Classification



Classification Scheme of GPCRs. Class A (Rhodopsin-like), Class B (Secretin-like), Class C (Glutamate Receptor-like), Others (Adhesion (33), Frizzled (11), Taste type-2 (25), unclassified (23)).

The exact size of the GPCR superfamily is unknown but nearly 800 different human genes (or  $\approx 4\%$  of the entire protein-coding genome) have been predicted from genome sequence analysis. Although numerous classification schemes have been proposed, the superfamily is classically divided into three main classes (A, B, and C) with no detectable shared sequence homology between classes. The largest class by far is class A, which accounts for nearly 85% of the GPCR genes. Of class A GPCRs, over half of these are predicted to encode olfactory receptors while the remaining receptors are liganded by known endogenous compounds or are classified as orphan receptors. Despite the lack of sequence homology between classes, all GPCRs share a common structure and mechanism of signal transduction.

In all, GPCRs can be grouped into 6 classes based on sequence homology and functional similarity:

- Class A (or 1) (Rhodopsin-like)
- Class B (or 2) (Secretin receptor family)
- Class C (or 3) (Metabotropic glutamate/pheromone)
- Class D (or 4) (Fungal mating pheromone receptors)
- Class E (or 5) (Cyclic AMP receptors)
- Class F (or 6) (Frizzled/Smoothed)

The very large rhodopsin A group has been further subdivided into 19 subgroups (A1-A19). More recently, an alternative classification system called GRAFS (Glutamate, Rhodopsin, Adhesion, Frizzled/Taste2, Secretin) has been proposed.

The human genome encodes thousands of G protein-coupled receptors, about 350 of which detect hormones, growth factors, and other endogenous ligands. Approximately 150 of the GPCRs found in the human genome have unknown functions.

Some web-servers and bioinformatics prediction methods have been used for predicting the classification of GPCRs according to their amino acid sequence alone, by means of the pseudo amino acid composition approach.

### ***Physiological roles***

GPCRs are involved in a wide variety of physiological processes. Some examples of their physiological roles include:

1. the visual sense: the opsins use a photoisomerization reaction to translate electromagnetic radiation into cellular signals. Rhodopsin, for example, uses the conversion of *11-cis*-retinal to *all-trans*-retinal for this purpose
2. the sense of smell: receptors of the olfactory epithelium bind odorants (olfactory receptors) and pheromones (vomeronasal receptors)
3. behavioral and mood regulation: receptors in the mammalian brain bind several different neurotransmitters, including serotonin, dopamine, GABA, and glutamate
4. regulation of immune system activity and inflammation: chemokine receptors bind ligands that mediate intercellular communication between cells of the immune system; receptors such as histamine receptors bind inflammatory mediators and engage target cell types in the inflammatory response
5. autonomic nervous system transmission: both the sympathetic and parasympathetic nervous systems are regulated by GPCR pathways, responsible for control of many automatic functions of the body such as blood pressure, heart rate, and digestive processes
6. cell density sensing: A novel GPCR role in regulating cell density sensing.

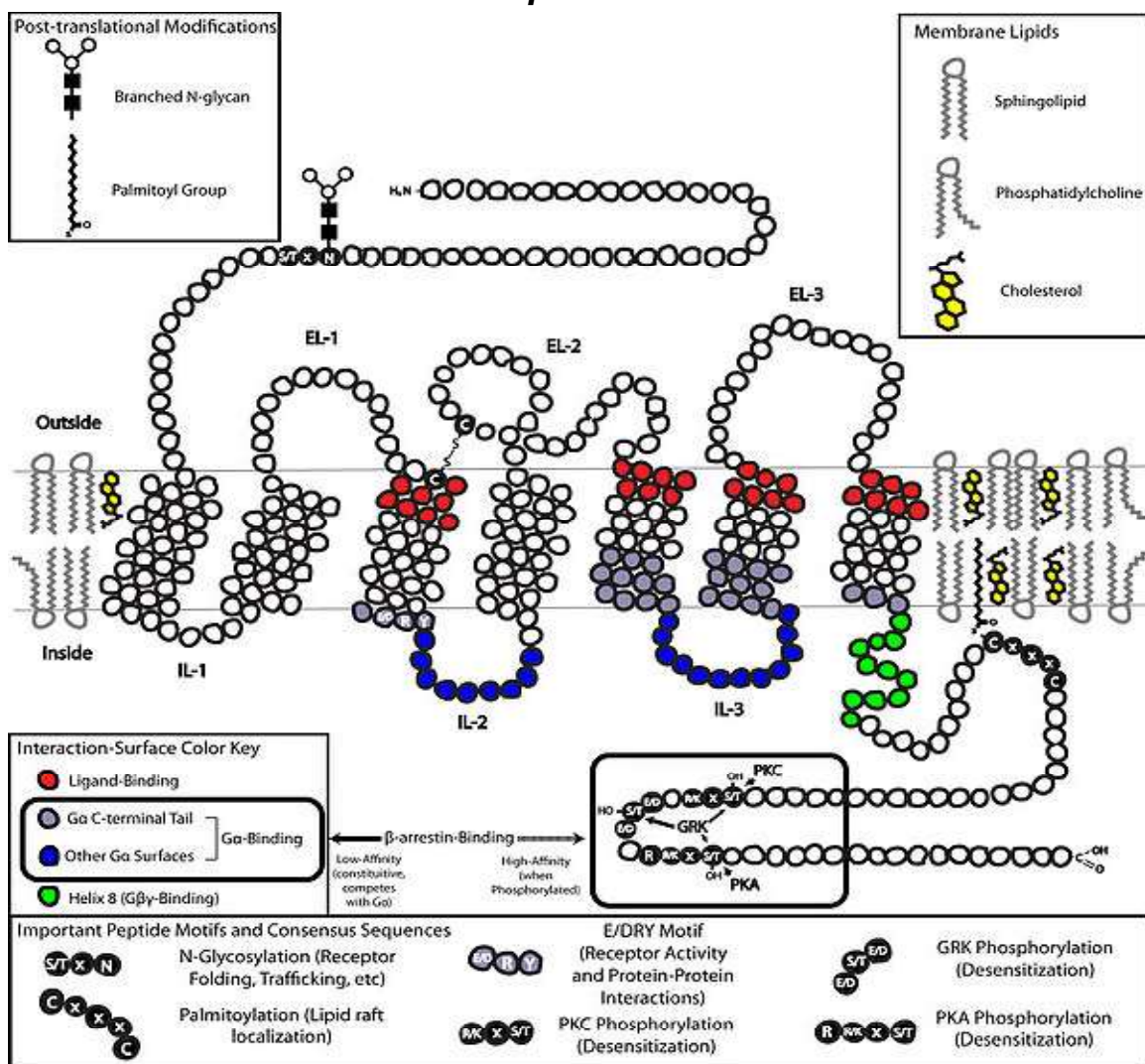
### ***Receptor structure***

GPCRs are integral membrane proteins that possess seven membrane-spanning domains or transmembrane helices. The extracellular parts of the receptor can be glycosylated. These extracellular loops also contain two highly-conserved cysteine residues that form disulfide bonds to stabilize the receptor structure. Some seven-transmembrane helix proteins (channelrhodopsin) that resemble GPCRs may contain ion channels, within their protein.

Early structural models for GPCRs were based on their weak analogy to bacteriorhodopsin, for which a structure had been determined by both electron diffraction (PDB 2BRD, 1AT9) and X ray-based crystallography (1AP9). In 2000, the first crystal

structure of a mammalian GPCR, that of bovine rhodopsin (1F88), was solved. While the main feature, the seven transmembrane helices, is conserved, the relative orientation of the helices differ significantly from that of bacteriorhodopsin. In 2007, the first structure of a human GPCR was solved (2R4R, 2R4S). This was followed immediately by a higher resolution structure of the same receptor (2RH1). This human  $\beta_2$ -adrenergic receptor GPCR structure, proved to be highly similar to the bovine rhodopsin in terms of the relative orientation of the seven-transmembrane helices. However the conformation of the second extracellular loop is entirely different between the two structures. Since this loop constitutes the "lid" that covers the top of the ligand binding site, this conformational difference highlights the difficulties in constructing homology models of other GPCRs based only on the rhodopsin structure.

### Structure-function relationships



Two-dimensional schematic of a generic GPCR set in a Lipid Raft.

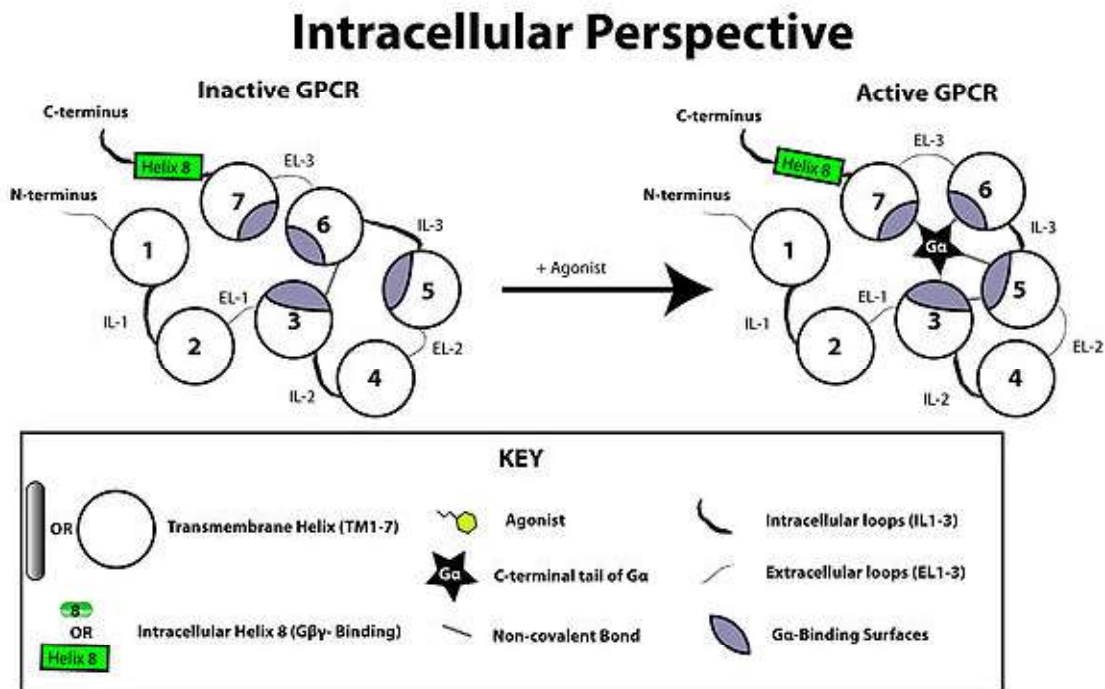
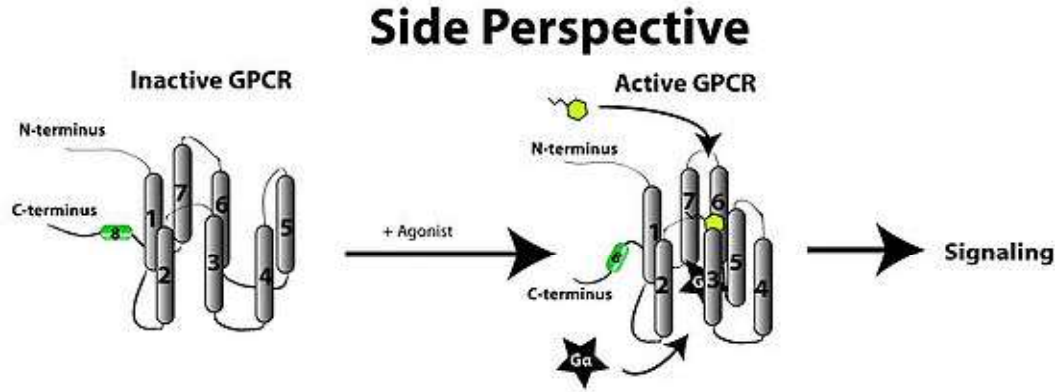
Structurally GPCRs are characterized by an extracellular N-terminus, followed by seven transmembrane (7-TM)  $\alpha$ -helices (TM-1 to TM-7) connected by three intracellular (IL-1 to IL-3) and three extracellular loops (EL-1 to EL-3), and finally an intracellular C-terminus. The GPCR arranges itself into a tertiary structure resembling a barrel, with the seven transmembrane helices forming a cavity within the plasma membrane which serves as a ligand-binding domain that is often covered by EL-2. Ligands may also bind elsewhere, however, as is the case for bulkier ligands (e.g., proteins or large peptides) which instead interact with the extracellular loops, or, as illustrated by the class C metabotropic glutamate receptors (mGluRs), the N-terminal tail. The class C GPCRs are distinguished by their large N-terminal tail, which also contains a ligand-binding domain. Upon glutamate-binding to an mGluR, the N-terminal tail undergoes a conformational change that leads to its interaction with the residues of the extracellular loops and TM domains. The eventual effect of all three types of agonist-induced activation is a change in the relative orientations of the TM helices (likened to a twisting motion) leading to a wider intracellular surface and “revelation” of residues of the intracellular helices and TM domains crucial to signal transduction function (i.e., G-protein coupling). Inverse agonists and antagonists may also bind to a number of different sites, but the eventual effect must be prevention of this TM helix reorientation.

The structure of the N- and C-terminal tails of GPCRs may also serve important functions beyond ligand-binding. In particular, the C-terminus often contains serine (Ser) or threonine (Thr) residues that, when phosphorylated, increase the affinity of the intracellular surface for the binding of scaffolding proteins called  $\beta$ -arrestins ( $\beta$ -arr). Once bound,  $\beta$ -arrestins both sterically prevent G-protein coupling and may recruit other proteins leading to the creation of signaling complexes involved in extracellular-signal regulated kinase (ERK) pathway activation or receptor endocytosis (internalization). As the phosphorylation of these Ser and Thr residues often occurs as a result of GPCR activation, the  $\beta$ -arr-mediated G-protein-decoupling and internalization of GPCRs are important mechanisms of desensitization.

A final common structural theme amongst GPCRs is palmitoylation of one or more sites of the C-terminal tail or the intracellular loops. Palmitoylation is the covalent modification of cysteine (Cys) residues via addition of hydrophobic acyl groups, and has the effect of targeting the receptor to cholesterol- and sphingolipid-rich microdomains of the plasma membrane called lipid rafts. As many of the downstream transducer and effector molecules of GPCRs (including those involved in negative feedback pathways) are also targeted to lipid rafts, this has the effect of facilitating rapid receptor signaling.

GPCRs respond to extracellular signals mediated by a huge diversity of agonists, ranging from proteins to biogenic amines to protons, but all transduce this signal via a mechanism of G-protein coupling. This is made possible by virtue of a guanine-nucleotide exchange factor (GEF) domain primarily formed by a combination of IL-2 and IL-3 along with adjacent residues of the associated TM helices.

## Mechanism



Cartoon depicting the basic concept of GPCR Conformational Activation. Ligand binding disrupts an ionic lock between the E/DRY motif of TM-3 and acidic residues of TM-6. As a result the GPCR reorganizes to allow activation of G-alpha proteins. The side perspective is a view from above and to the side of the GPCR as it is set in the plasma membrane (the membrane lipids have been omitted for clarity). The intracellular perspective shows the view looking up at the plasma membrane from inside the cell.

The G protein-coupled receptor is activated by an external signal in the form of a ligand or other signal mediator. This creates a conformational change in the receptor, causing activation of a G protein. Further effect depends on the type of G protein.

## Ligand binding

GPCRs include receptors for sensory signal mediators (e.g., light and olfactory stimulatory molecules); adenosine, bombesin, bradykinin, endothelin,  $\gamma$ -aminobutyric acid (GABA), hepatocyte growth factor (HGF), melanocortins, neuropeptide Y, opioid peptides, opsins, somatostatin, tachykinins, members of the vasoactive intestinal peptide family, and vasopressin; biogenic amines (e.g., dopamine, epinephrine, norepinephrine, histamine, glutamate (metabotropic effect), glucagon, acetylcholine (muscarinic effect), and serotonin); chemokines; lipid mediators of inflammation (e.g., prostaglandins, prostanoids, platelet-activating factor, and leukotrienes); and peptide hormones (e.g., calcitonin, C5a anaphylatoxin, follicle-stimulating hormone (FSH), gonadotropin-releasing hormone (GnRH), neurokinin, thyrotropin-releasing hormone (TRH), and oxytocin). GPCRs that act as receptors for stimuli that have not yet been identified are known as orphan receptors.

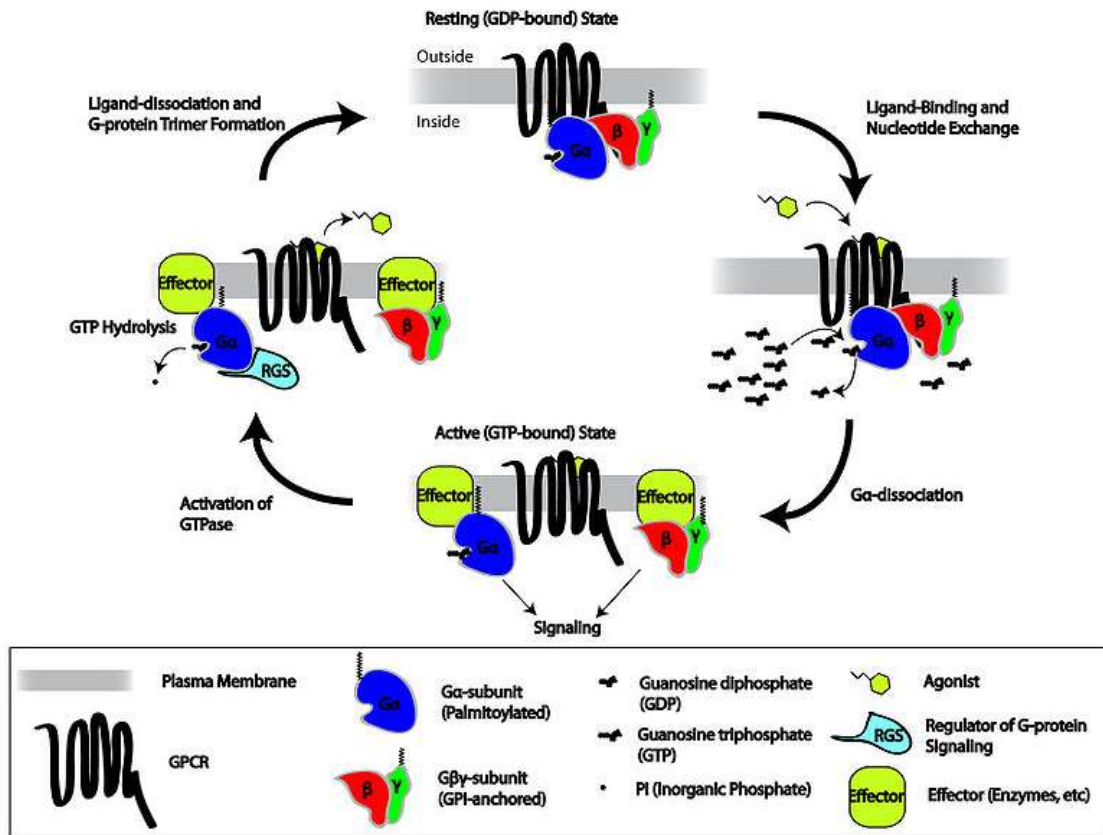
Whereas, in other types of receptors that have been studied, wherein ligands bind externally to the membrane, the ligands of GPCRs typically bind within the transmembrane domain. However, protease-activated receptors are activated by cleavage of part of their extracellular domain.

## Conformational change

The transduction of the signal through the membrane by the receptor is not completely understood. It is known that the inactive G protein is bound to the receptor in its inactive state. Once the ligand is recognized, the receptor shifts conformation and, thus, mechanically activates the G protein, which detaches from the receptor. The receptor can now either activate another G protein or switch back to its inactive state. This is an overly simplistic explanation, but suffices to convey the overall set of events.

It is believed that a receptor molecule exists in a conformational equilibrium between active and inactive biophysical states. The binding of ligands to the receptor may shift the equilibrium toward the active receptor states. Three types of ligands exist: Agonists are ligands that shift the equilibrium in favour of active states; inverse agonists are ligands that shift the equilibrium in favour of inactive states; and neutral antagonists are ligands that do not affect the equilibrium. It is not yet known how exactly the active and inactive states differ from each other.

## G-protein activation/deactivation cycle

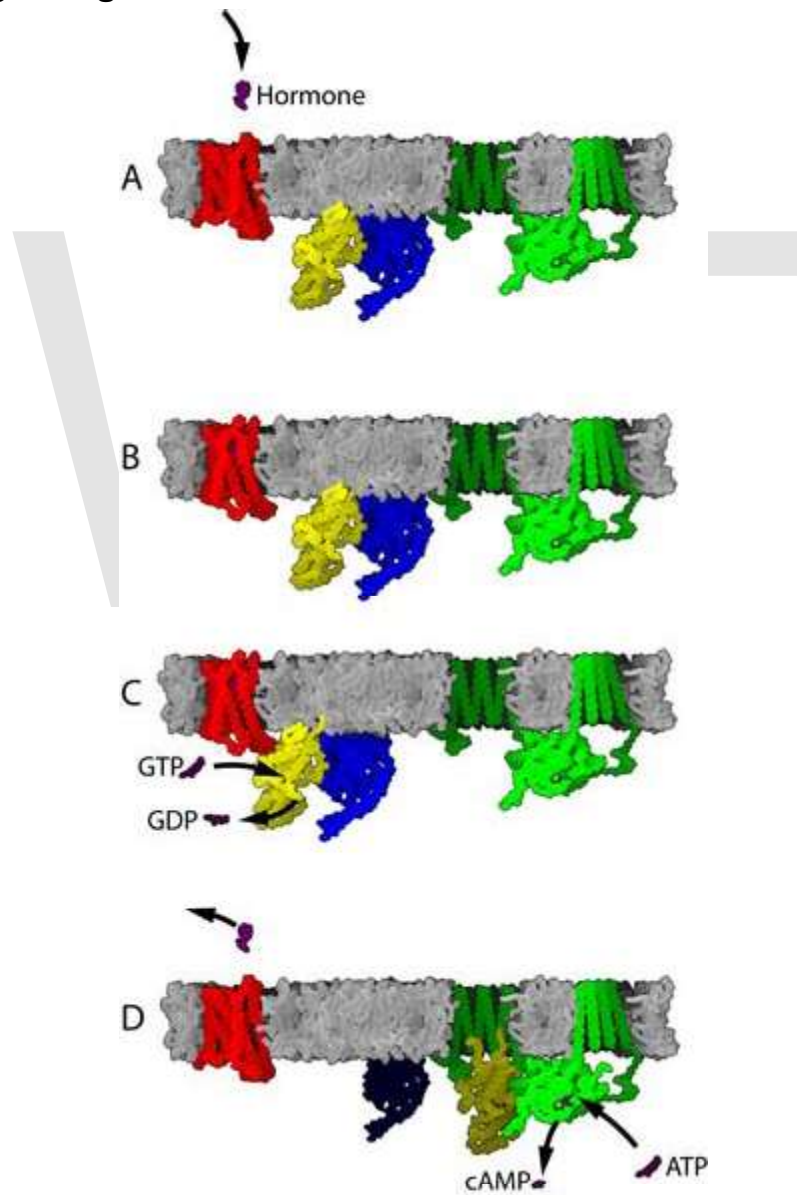


Cartoon depicting the Heterotrimeric G-protein activation/deactivation cycle in the context of GPCR signaling

When the receptor is inactive, the GEF domain may be bound to an also inactive  $\alpha$ -subunit of a heterotrimeric G-protein. These “G-proteins” are a trimer of  $\alpha$ ,  $\beta$ , and  $\gamma$  subunits (known as  $G\alpha$ ,  $G\beta$ , and  $G\gamma$ , respectively) which is rendered inactive when reversibly bound to Guanosine diphosphate (GDP) (or alternatively, no guanine nucleotide) but active when bound to Guanosine triphosphate (GTP). Upon receptor activation, the GEF domain, in turn, allosterically activates the G-protein by facilitating the exchange of a molecule of GDP for GTP at the G-protein's  $\alpha$ -subunit. The cell maintains a 10:1 ratio of cytosolic GTP:GDP so exchange for GTP is ensured. At this point, the subunits of the G-protein dissociate from the receptor, as well as each other, to yield a  $G\alpha$ -GTP monomer and a tightly interacting  $G\beta\gamma$  dimer, which are now free to modulate the activity of other intracellular proteins. The extent to which they may diffuse, however, is limited due to the palmitoylation of  $G\alpha$  and the presence of a molecule of Glycosylphosphatidylinositol (GPI) that has been covalently added to the C-termini of  $G\gamma$ . The phosphatidylinositol moiety of the GPI-linkage contains two hydrophobic acyl groups that anchor any GPI-linked proteins (e.g.  $G\beta\gamma$ ) to the plasma membrane, and also, to some extent, to the local lipid raft. (Compare this to the effect of palmitoylation on GPCR localization discussed above)

Because  $G\alpha$  also has slow  $GTP \rightarrow GDP$  hydrolysis capability, the inactive form of the  $\alpha$ -subunit ( $G\alpha$ -GDP) is eventually regenerated, thus allowing reassociation with a  $G\beta\gamma$  dimer to form the “resting” G-protein which can again bind to a GPCR and await activation. The rate of GTP hydrolysis is often accelerated due to the actions of another family of allosteric modulating proteins called Regulators of G-protein Signaling, or RGS proteins, which are a type of GTPase-Activating Protein, or GAP. In fact, many of the primary effector proteins (e.g. adenylate cyclases) that become activated/inactivated upon interaction with  $G\alpha$ -GTP also have GAP activity. Thus, even at this early stage in the process, GPCR-initiated signaling has the capacity for self-termination.

### ***GPCR signaling***



G-protein-coupled receptor mechanism

If a receptor in an active state encounters a G protein, it may activate it. Some evidence suggests that receptors and G proteins are actually pre-coupled. For example, binding of G proteins to receptors affects the receptor's affinity for ligands. Activated G proteins are bound to GTP.

Further signal transduction depends on the type of G protein. The enzyme adenylate cyclase is an example of a cellular protein that can be regulated by a G protein, in this case the G protein  $G_s$ . Adenylate cyclase activity is activated when it binds to a subunit of the activated G protein. Activation of adenylate cyclase ends when the G protein returns to the GDP-bound state.

Adenylate cyclases (of which 9 membrane bound and one cytosolic forms are known in humans) may also be activated or inhibited in other ways (e.g.  $Ca^{2+}$ /Calmodulin binding) and that this can modify the activity of these enzymes in an additive or synergistic fashion along with the G proteins.

The signaling pathways activated through a GPCR are limited by the primary sequence and tertiary structure of the GPCR itself but ultimately determined by the particular conformation stabilized by a particular ligand, as well as the availability of transducer molecules. Currently, GPCRs are considered to utilize two primary types of transducers: G-proteins and  $\beta$ -arrestins. Because  $\beta$ -arr's only have high affinity to the phosphorylated form of most GPCRs, the majority of signaling is ultimately dependent upon G-protein activation. However, the possibility for interaction does allow for G-protein independent signaling to occur.

### **G-protein-dependent signaling**

There are three main G-protein-mediated signaling pathways, mediated by four sub-classes of G-proteins distinguished from each other by sequence homology ( $G_{as}$ ,  $G_{ai/o}$ ,  $G_{aq/11}$ , and  $G_{\alpha 12/13}$ ). Each sub-class of G-protein consists of multiple proteins, each the product of multiple genes and/or splice variations that may imbue them with differences ranging from subtle to distinct with regard to signaling properties, but in general they appear to be reasonably grouped into four classes. Because the signal transducing properties of the various possible  $\beta\gamma$  combinations do not appear to radically differ from one another, these classes are defined according to the isoform of their  $\alpha$ -subunit.

While most GPCRs are capable of activating more than one  $G\alpha$ -subtype, they also show a preference for one subtype over another. When the subtype activated depends on the ligand that is bound to the GPCR, this is called functional selectivity (also known as agonist-directed trafficking, or conformation specific agonism). However, the binding of any single particular agonist may also initiate activation of multiple different G-proteins, as it may be capable of stabilizing more than one conformation of the GPCR's GEF domain, even over the course of a single interaction. Additionally, a conformation that preferably activates one isoform of  $G\alpha$  may activate another if the preferred is less available. Furthermore, feedback pathways may result in receptor modifications (e.g. phosphorylation) that alter the G-protein preference. Regardless of these various nuances,

the GPCR's preferred coupling partner is usually defined according to the G-protein most obviously activated by the endogenous ligand under most physiological and/or experimental conditions.

### **G<sub>α</sub> signaling**

1. The effector of both the G<sub>as</sub> and G<sub>ai/o</sub> pathways is the Cyclic-adenosine monophosphate (cAMP) generating enzyme Adenylate Cyclase, or AC. While there are ten different AC gene products in mammals, each with subtle differences in tissue distribution and/or function, all catalyze the conversion of cytosolic Adenosine Triphosphate (ATP) to cAMP, and all are directly stimulated by G-proteins of the G<sub>as</sub> class. Conversely, interaction with G<sub>α</sub> subunits of the G<sub>ai/o</sub> type inhibits AC from generating cAMP. Thus, a GPCR coupled to G<sub>as</sub> will counteract the actions of a GPCR coupled to G<sub>ai/o</sub>, and vice versa. The level of cytosolic cAMP may then determine the activity of various ion channels as well as members of the ser/thr specific Protein Kinase A (PKA) family. Thus cAMP is considered a second messenger and PKA a secondary effector.
2. The effector of the G<sub>αq/11</sub> pathway is Phospholipase C-β (PLCβ), which catalyzes the cleavage of membrane-bound phosphatidylinositol 4,5-bisphosphate (PIP<sub>2</sub>) into the second messengers inositol (1,4,5) triphosphate (IP<sub>3</sub>) and diacylglycerol (DAG). IP<sub>3</sub> acts on IP<sub>3</sub> receptors found in the membrane of the endoplasmic reticulum (ER) to elicit Ca<sup>2+</sup> release from the ER, while DAG diffuses along the plasma membrane where it may activate any membrane localized forms of a second ser/thr kinase called Protein Kinase C (PKC). Since many isoforms of PKC are also activated by increases in intracellular Ca<sup>2+</sup>, both these pathways can also converge on each other to signal through the same secondary effector. Elevated intracellular Ca<sup>2+</sup> also binds and allosterically activates proteins called Calmodulins, which in turn go on to bind and allosterically activate enzymes such as Ca<sup>2+</sup>/Calmodulin-dependant Kinases (CAMKs).
3. The effectors of the G<sub>α12/13</sub> pathway are three RhoGEFs (p115-RhoGEF, PDZ-RhoGEF, and LARG), which, when bound to G<sub>α12/13</sub> allosterically activate the cytosolic small GTPase, Rho. Once bound to GTP, Rho can then go on to activate various proteins responsible for cytoskeleton regulation such as Rho-kinase (ROCK). Most GPCRs that couple to G<sub>α12/13</sub> also couple to other sub-classes, often G<sub>αq/11</sub>.

### **Gβγ signaling**

The above descriptions ignore the effects of Gβγ-signalling, which can also be important, particularly in the case of activated G<sub>ai/o</sub>-coupled GPCRs. The primary effectors of Gβγ are various ion channels, such as G-protein-regulated Inwardly Rectifying K<sup>+</sup> channels (GIRKs), P/Q- and N-type voltage-gated Ca<sup>2+</sup> Channels, as well as some isoforms of AC and PLC, along with some Phosphoinositide-3-Kinase (PI3K) isoforms.

## G-Protein-independent signaling

Although they are classically thought of working only together, GPCRs may signal through G-protein-independent mechanisms, and heterotrimeric G-proteins may play functional roles independent of GPCRs. GPCRs may signal independently through many proteins already mentioned for their roles in G-protein-dependent signaling such as  $\beta$ -arrests, GRKs, and Srcs. Additionally, further scaffolding proteins involved in subcellular localization of GPCRs (e.g. PDZ-domain-containing proteins) may also act as signal transducers. Most often the effector is a member of the MAPK family.

### Examples

In the late 1990s, evidence began accumulating to suggest that some GPCRs are able to signal without G proteins. The ERK2 mitogen-activated protein kinase, a key signal transduction mediator downstream of receptor activation in many pathways, has been shown to be activated in response to cAMP-mediated receptor activation in the slime mold *D. discoideum* despite the absence of the associated G protein  $\alpha$ - and  $\beta$ -subunits.

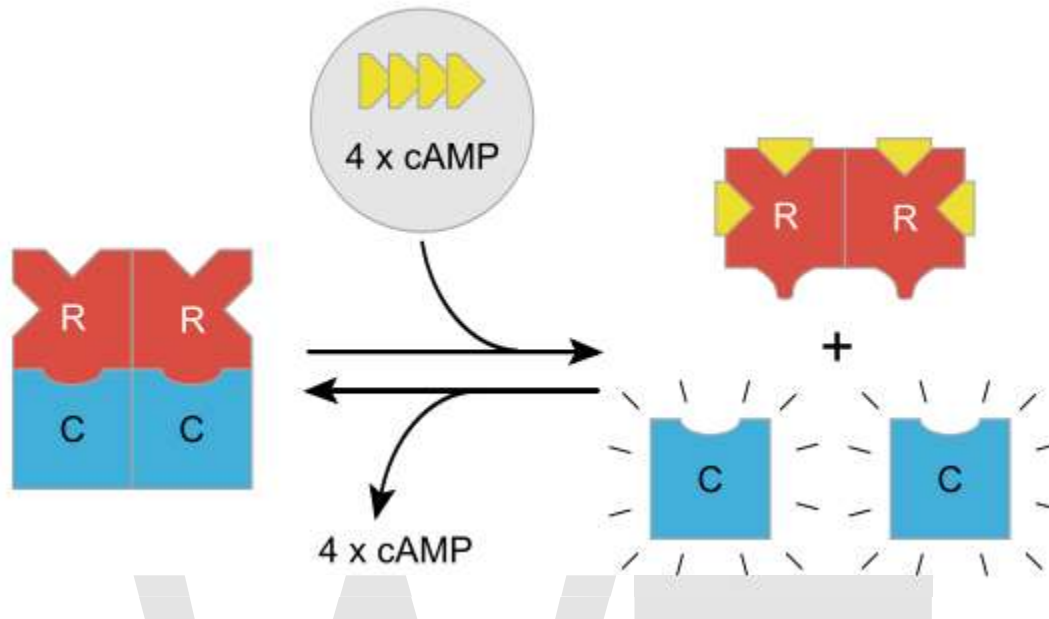
In mammalian cells, the much-studied  $\beta_2$ -adrenoceptor has been demonstrated to activate the ERK2 pathway after arrestin-mediated uncoupling of G-protein-mediated signaling. Therefore it seems likely that some mechanisms previously believed to be purely related to receptor desensitisation are actually examples of receptors switching their signaling pathway rather than simply being switched off.

In kidney cells, the bradykinin receptor B2 has been shown to interact directly with a protein tyrosine phosphatase. The presence of a tyrosine-phosphorylated ITIM (immunoreceptor tyrosine-based inhibitory motif) sequence in the B2 receptor is necessary to mediate this interaction and subsequently the antiproliferative effect of bradykinin.

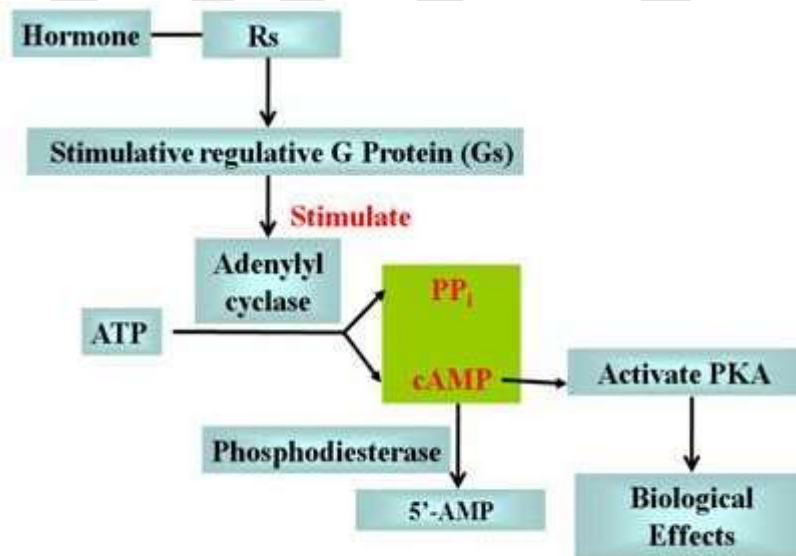
### GPCR-independent signaling by heterotrimeric G-proteins

Although it is a relatively immature area of research, it appears that heterotrimeric G-proteins may also take part in non-GPCR signaling. There is evidence for roles as signal transducers in nearly all other types of receptor-mediated signaling, including integrins, receptor tyrosine kinases (RTKs), cytokine receptors (JAK/STATs), as well as modulation of various other “accessory” proteins such as GEFs, Guanine-nucleotide Dissociation Inhibitors (GDIs) and protein phosphatases. There may even be specific proteins of these classes whose primary function is as part of GPCR-independent pathways, termed Activators of G-protein Signalling (AGS). Both the ubiquity of these interactions and the importance of  $G\alpha$  vs.  $G\beta\gamma$  subunits to these processes are still unclear.

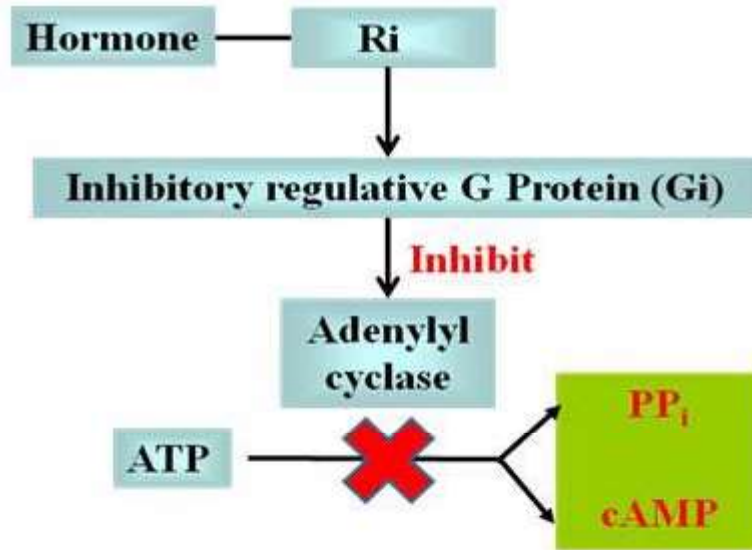
## Details of cAMP and PIP2 pathways



Activation effects of cAMP on Protein Kinase A



The effect of Rs and Gs in cAMP signal pathway



The effect of Ri and Gi in cAMP signal pathway

There are two principal signal transduction pathways involving the G protein-linked receptors: cAMP signal pathway and Phosphatidylinositol signal pathway.

### **cAMP signal pathway**

The cAMP signal transduction contains 5 main characters: stimulative hormone receptor (Rs) or inhibitory hormone receptor (Ri) ; Stimulative regulative G-protein (Gs) or inhibitory regulative G-protein (Gi) ; Adenylyl cyclase; Protein Kinase A (PKA); and cAMP phosphodiesterase.

Stimulative hormone receptor (Rs) is a receptor that can bind with stimulative signal molecules, while inhibitory hormone (Ri) is a receptor that can bind with inhibitory signal molecules.

Stimulative regulative G-protein is a G protein-linked to stimulative hormone receptor (Rs) and its  $\alpha$  subunit upon activation could stimulate the activity of an enzyme or other intracellular metabolism. On the contrary, inhibitory regulative G-protein is linked to an inhibitory hormone receptor and its  $\alpha$  subunit upon activation could inhibit the activity of an enzyme or other intracellular metabolism.

The Adenylyl cyclase is a 12-transmembrane glucoprotein that catalyzes ATP to form cAMP with the help of cofactor  $Mg^{2+}$  or  $Mn^{2+}$ . The cAMP produced is a second messenger in cellular metabolism and is an allosteric activator to Protein kinase A.

Protein kinase A is an important enzyme in cell metabolism due to its ability to regulate cell metabolism by phosphorylating specific committed enzymes in the metabolic pathway. It can also regulate specific gene expression, cellular secretion, and membrane

permeability. The protein enzyme contains two catalytic subunits and two regulatory subunits. When there is no cAMP, the complex is inactive. When cAMP binds to the regulatory subunits, their conformation is altered, causing the dissociation of the regulatory subunits, which activates protein kinase A and allows further biological effects.

cAMP phosphodiesterase is an enzyme that can degrade cAMP to 5'-AMP, which will terminate the signal.

## **Phosphatidylinositol signal pathway**

In the phosphatidylinositol signal pathway, the extracellular signal molecule binds with the G-protein receptor ( $G_q$ ) on the cell surface and activates phospholipase C, which is located on the plasma membrane. The lipase hydrolyzes phosphatidylinositol 4,5-bisphosphate (PIP<sub>2</sub>) into two second messengers: Inositol 1,4,5-triphosphate (IP<sub>3</sub>) and Diacylglycerol (DAG). IP<sub>3</sub> binds with the receptor in the membrane of the smooth endoplasmic reticulum and mitochondria, help open the  $Ca^{2+}$  channel. DAG will help activate Protein Kinase C (PKC), which phosphorylates many other proteins, changing their catalytic activities, leading to cellular responses. The effects of  $Ca^{2+}$  is also remarkable: it cooperates with DAG in activating PKC and can activate CaM kinase pathway, in which calcium modulated protein calmodulin (CaM) binds  $Ca^{2+}$ , undergoes a change in conformation, and activates CaM kinase II, which has unique ability to increase its binding affinity to CaM by autophosphorylation, making CaM unavailable for the activation of other enzymes. The kinase then phosphorylates target enzymes, regulating their activities. The two signal pathways are connected together by  $Ca^{2+}$ -CaM, which is also a regulatory subunit of adenylyl cyclase and phosphodiesterase in cAMP signal pathway.

## **Receptor regulation**

GPCRs become desensitized when exposed to their ligand for a prolonged period of time. There are two recognized forms of desensitization: 1) homologous desensitization, in which the activated GPCR is downregulated; and 2) heterologous desensitization, wherein the activated GPCR causes downregulation of a different GPCR. The key reaction of this downregulation is the phosphorylation of the intracellular (or cytoplasmic) receptor domain by protein kinases.

## **Phosphorylation by cAMP-dependent protein kinases**

Cyclic AMP-dependent protein kinases (protein kinase A) are activated by the signal chain coming from the G protein (that was activated by the receptor) via adenylyl cyclase and cyclic AMP (cAMP). In a *feedback mechanism*, these activated kinases phosphorylate the receptor. The longer the receptor remains active, the more kinases are activated, the more receptors are phosphorylated. In  $\beta_2$ -adrenoceptors, this phosphorylation results in the switching of the coupling from the  $G_s$  class of G-protein to

the G<sub>i</sub> class. cAMP-dependent PKA mediated phosphorylation can cause heterologous desensitisation in receptors other than those activated.

## Phosphorylation by GRKs

The G protein-coupled receptor kinases (GRKs) are protein kinases that phosphorylate only active GPCRs.

Phosphorylation of the receptor can have two consequences:

1. *Translocation*: The receptor is, along with the part of the membrane it is embedded in, brought to the inside of the cell, where it is dephosphorylated within the acidic vesicular environment and then brought back. This mechanism is used to regulate long-term exposure, for example, to a hormone, by allowing resensitisation to follow desensitisation. Alternatively, the receptor may undergo lysosomal degradation, or remain internalised, where it is thought to participate in the initiation of signalling events, the nature of which depend on the internalised vesicle's subcellular localisation.
2. *Arrestin linking*: The phosphorylated receptor can be linked to *arrestin* molecules that prevent it from binding (and activating) G proteins, effectively switching it off for a short period of time. This mechanism is used, for example, with rhodopsin in retina cells to compensate for exposure to bright light. In many cases, arrestin binding to the receptor is a prerequisite for translocation. For example, beta-arrestin bound to  $\beta_2$ -adrenoreceptors acts as an adaptor for binding with clathrin, and with the beta-subunit of AP2 (clathrin adaptor molecules); thus the arrestin here acts as a scaffold assembling the components needed for clathrin-mediated endocytosis of  $\beta_2$ -adrenoreceptors.

## Mechanisms of GPCR signal termination

As mentioned above, G-proteins may terminate their own activation due to their intrinsic GTP→GDP hydrolysis capability. However, this reaction proceeds at a slow rate ( $\approx .02$  times/sec) and thus it would take around 50 seconds for any single G-protein to deactivate if other factors did not come into play. Indeed, there are around 30 isoforms of RGS proteins that, when bound to G $\alpha$  through their GAP domain, accelerate the hydrolysis rate to  $\approx 30$  times/sec. This 1500-fold increase in rate allows for the cell to respond to external signals with high speed, as well as spatial resolution due to limited amount of second messenger that can be generated and limited distance a G-protein can diffuse in .03 seconds. For the most part, the RGS proteins are promiscuous in their ability to activate G-proteins, while which RGS is involved in a given signaling pathway seems to be more determined by the tissue and GPCR involved than anything else. Additionally, RGS proteins have the additional function of increasing the rate of GTP-GDP exchange at GPCRs, (i.e. as a sort of co-GEF) further contributing to the time resolution of GPCR signaling.

In addition, the GPCR may be desensitized itself. This can occur as:

1. a direct result of ligand occupation, wherein the change in conformation allows recruitment of GPCR-Regulating Kinases (GRKs), which go on to phosphorylate various serine/threonine residues of IL-3 and the C-terminal tail. Upon GRK phosphorylation, the GPCR's affinity for  $\beta$ -arrestin ( $\beta$ -arrestin-1/2 in most tissues) is increased, at which point  $\beta$ -arrestin may bind and act to both sterically hinder G-protein coupling as well as initiate the process of receptor internalization through clathrin-mediated endocytosis. Because only the liganded receptor is desensitized by this mechanism, it is called homologous desensitization.
2. Alternatively, the affinity for  $\beta$ -arr may be increased in a ligand occupation and GRK-independent manner through phosphorylation of different ser/thr sites (but also of IL-3 and the C-terminal tail) by PKC and PKA. These phosphorylations are often sufficient to impair G-protein coupling on their own as well.
3. Thirdly, PKC/PKA may instead phosphorylate GRKs which can also lead to GPCR phosphorylation and  $\beta$ -arrestin binding in an occupation-independent manner. These latter two mechanisms allow for desensitization of one GPCR due to the activities of others, or heterologous desensitization. GRKs may also have GAP domains and so may contribute to inactivation through non-kinase mechanisms as well. A combination of these mechanisms may also occur.

Once  $\beta$ -arrestin is bound to a GPCR it undergoes a conformational change allowing it to serve as a scaffolding protein for an adaptor complex termed AP-2, which in turn recruits another protein called clathrin. If enough receptors in the local area recruit clathrin in this manner, they aggregate and the membrane buds inwardly as a result of interactions between the molecules of clathrin, in a process called opsonization. Once the pit has been pinched off the plasma membrane due to the actions of two other proteins called amphiphysin and dynamin, it is now an endocytic vesicle. At this point the adapter molecules and clathrin have dissociated and the receptor will be trafficked either back to the plasma membrane or targeted to lysosomes for degradation.

At any point in this process, the  $\beta$ -arrestins may also be recruiting other proteins such as the non-receptor tyrosine kinase (nRTK), c-SRC, which may initiate activation of ERK1/2, or other mitogen-activated protein kinase (MAPK) signaling through, for example, phosphorylation of the small GTP-ase, Ras, or recruit the proteins of the ERK cascade directly (i.e. Raf-1, MEK, ERK-1/2) at which point signaling is initiated due to their close proximity to one another. Another target of c-SRC are the dynamin molecules involved in endocytosis. Dynamins polymerize around the neck of an incoming vesicle, and their phosphorylation by c-SRC provides the energy necessary for the conformational change allowing the final “pinching off” from the membrane.

## **GPCR downregulation**

Receptor desensitization is mediated through a combination phosphorylation,  $\beta$ -arr binding, and endocytosis as described above. Downregulation occurs when endocytosed receptor is embedded in an endosome that is trafficked to merge with an organelle called

a lysosome. Because lysosomal membranes are rich in proton pumps, their interiors have low pH ( $\approx 4.8$  vs. the  $\text{pH} \approx 7.2$  cytosol) which acts to denature the GPCRs. Additionally, lysosomes contain many degradative enzymes, including proteases, which can only function at such low pH, and so the peptide bonds joining the residues of the GPCR together may be cleaved. Whether or not a given receptor is trafficked to a lysosome, detained in endosomes, or trafficked back to the plasma membrane depends on a variety of factors, including receptor type and magnitude of the signal.

### ***Receptor oligomerization***

It is generally accepted that G-protein-coupled receptors can form heteromers such as homo- and heterodimers as well as more complex oligomeric structures, and indeed heterodimerization has been shown to be essential for the function of receptors such as the metabotropic GABA(B) receptors. However, it is presently unproven that true heterodimers exist. Present biochemical and physical techniques lack the resolution to differentiate between distinct homodimers assembled into an oligomer or true 1:1 heterodimers. It is also unclear what the functional significance of oligomerization might be, although it is thought that the phenomenon may contribute to the pharmacological heterogeneity of GPCRs in a manner not previously anticipated. This is an actively-studied area in GPCR research.

The best-studied example of receptor oligomerisation are the metabotropic GABA<sub>B</sub> receptors. These receptors are formed by heterodimerization of GABA<sub>B</sub>R1 and GABA<sub>B</sub>R2 subunits. Expression of the GABA<sub>B</sub>R1 without the GABA<sub>B</sub>R2 in heterologous systems leads to retention of the subunit in the endoplasmic reticulum. Expression of the GABA<sub>B</sub>R2 subunit alone, meanwhile, leads to surface expression of the subunit, although with no functional activity (*i.e.*, the receptor does not bind agonist and cannot initiate a response following exposure to agonist). Expression of the two subunits together leads to plasma membrane expression of functional receptor. It has been shown that GABA<sub>B</sub>R2 binding to GABA<sub>B</sub>R1 causes masking of a retention signal of functional receptors.

### **Dictyostelium discoideum**

A novel GPCR containing a lipid kinase domain has recently been identified in *Dictyostelium discoideum* that regulates cell density sensing.

## Chapter 4

# Apoptosis



Apoptosis increasing from normal cells (top) to apoptotic ones (bottom).

**Apoptosis** is the process of programmed cell death (PCD) that may occur in multicellular organisms. Biochemical events lead to characteristic cell changes (morphology) and death. These changes include blebbing, loss of cell membrane asymmetry and attachment, cell shrinkage, nuclear fragmentation, chromatin condensation, and chromosomal DNA fragmentation. Apoptosis differs from necrosis, in which the cellular debris can damage the organism.

In contrast to necrosis, which is a form of traumatic cell death that results from acute cellular injury, apoptosis, in general, confers advantages during an organism's life cycle. For example, the differentiation of fingers and toes in a developing human embryo occurs because cells between the fingers apoptose; the result is that the digits are separate. Between 50 and 70 billion cells die each day due to apoptosis in the average human adult. For an average child between the ages of 8 and 14, approximately 20 billion to 30 billion cells die a day.

Research in and around apoptosis has increased substantially since the early 1990s. In addition to its importance as a biological phenomenon, defective apoptotic processes have been implicated in an extensive variety of diseases. Excessive apoptosis causes atrophy, such as in ischemic damage, whereas an insufficient amount results in uncontrolled cell proliferation, such as cancer.

### ***Discovery and etymology***

German scientist Carl Vogt was first to describe the principle of apoptosis in 1842. In 1885, anatomist Walther Flemming delivered a more precise description of the process of programmed cell death. However, it was not until 1965 that the topic was resurrected. While studying tissues using electron microscopy, John Foxton Ross Kerr at University of Queensland was able to distinguish apoptosis (Greek: *apo* - from/off/without, *ptosis* - falling) from traumatic cell death. Following the publication of a paper describing the phenomenon, Kerr was invited to join Alastair R Currie, as well as Andrew Wyllie, who was Currie's graduate student, at University of Aberdeen. In 1972, the trio published a seminal article in the British Journal of Cancer. Kerr had initially used the term programmed cell necrosis, but in the article, the process of natural cell death was called *apoptosis*. Kerr, Wyllie and Currie credited James Cormack, a professor of Greek language at University of Aberdeen, with suggesting the term apoptosis. Kerr received the Paul Ehrlich and Ludwig Darmstaedter Prize on March 14, 2000, for his description of apoptosis. He shared the prize with Boston biologist Robert Horvitz.. The 2002 Nobel Prize in Medicine was awarded to Sydney Brenner, Horvitz and John E. Sulston for their work regarding apoptosis.

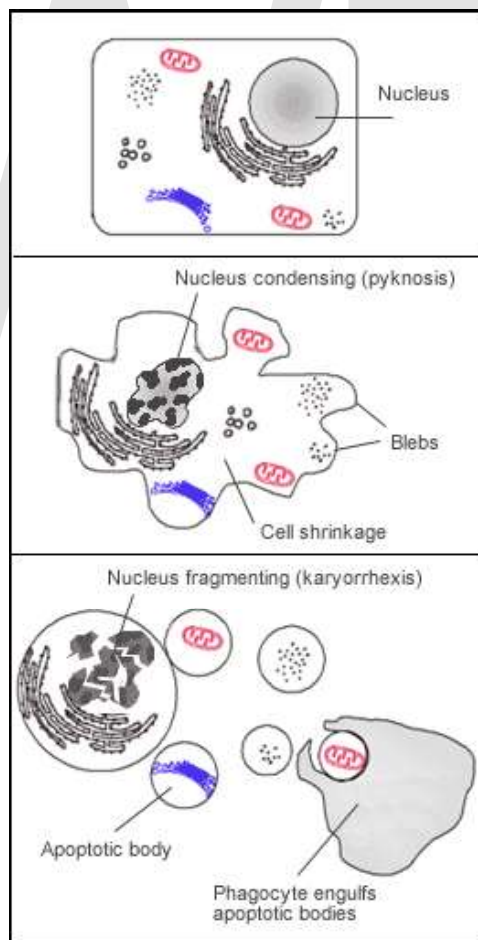
In Greek, apoptosis translates to the "dropping off" of petals or leaves from plants or trees. Cormack, professor of Greek language, reintroduced the term for medical use as it had a medical meaning for the Greeks over two thousand years before. Hippocrates used the term to mean "the falling off of the bones". Galen extended its meaning to "the dropping of the scabs". Cormack was no doubt aware of this usage when he suggested the name. Debate continues over the correct pronunciation, with opinion divided between a

pronunciation with the second *p* silent and the second *p* pronounced, as in the original Greek. In English, the *p* of the Greek *-pt-* consonant cluster is typically silent at the beginning of a word (e.g. pterodactyl, Ptolemy), but articulated when used in combining forms preceded by a vowel, as in helicopter or the orders of insects: diptera, lepidoptera, etc.

In the original Kerr Wyllie and Currie paper, *British Journal of Cancer*, 1972 Aug;26(4):239-57, there is a footnote regarding the pronunciation:

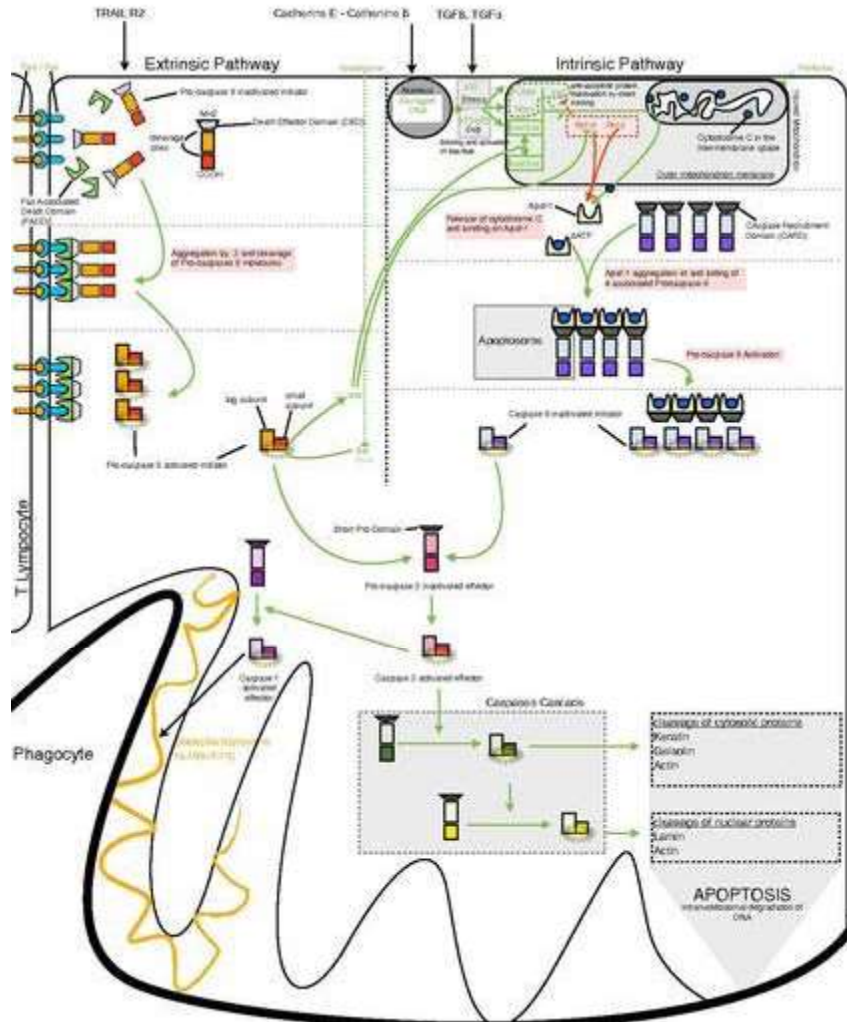
"We are most grateful to Professor James Cormack of the Department of Greek, University of Aberdeen, for suggesting this term. The word "apoptosis" (Greek spelling of apoptosis) is used in Greek to describe the "dropping off" or "falling off" of petals from flowers, or leaves from trees. To show the derivation clearly, we propose that the stress should be on the penultimate syllable, the second half of the word being pronounced like "ptosis" (with the "p" silent), which comes from the same root "to fall", and is already used to describe the drooping of the upper eyelid."

## Process



# Control of the Apoptosis Mecanisms

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## Control Of The Apoptosis Mecanisms

The process of apoptosis is controlled by a diverse range of cell signals, which may originate either extracellularly (*extrinsic inducers*) or intracellularly (*intrinsic inducers*). Extracellular signals may include toxins, hormones, growth factors, nitric oxide or cytokines, that must either cross the plasma membrane or transduce to effect a response. These signals may positively (i.e., trigger) or negatively (i.e., repress, inhibit, or dampen) affect apoptosis. (Binding and subsequent initiation of apoptosis by a molecule is termed *positive induction*, whereas the active repression or inhibition of apoptosis by a molecule is termed *negative induction*.)

A cell initiates intracellular apoptotic signalling in response to a stress, which may bring about cell suicide. The binding of nuclear receptors by glucocorticoids, heat, radiation,

nutrient deprivation, viral infection, hypoxia and increased intracellular calcium concentration, for example, by damage to the membrane, can all trigger the release of intracellular apoptotic signals by a damaged cell. A number of cellular components, such as poly ADP ribose polymerase, may also help regulate apoptosis.

Before the actual process of cell death is precipitated by enzymes, apoptotic signals must cause regulatory proteins to initiate the apoptosis pathway. This step allows apoptotic signals to cause cell death, or the process to be stopped, should the cell no longer need to die. Several proteins are involved, but two main methods of regulation have been identified: targeting mitochondria functionality, or directly transducing the signal via adaptor proteins to the apoptotic mechanisms. Another extrinsic pathway for initiation identified in several toxin studies is an increase in calcium concentration within a cell caused by drug activity, which also can cause apoptosis via a calcium binding protease calpain.

## Mitochondrial regulation

The mitochondria are essential to multicellular life. Without them, a cell ceases to respire aerobically and quickly dies, a fact exploited by some apoptotic pathways. Apoptotic proteins that target mitochondria affect them in different ways. They may cause mitochondrial swelling through the formation of membrane pores, or they may increase the permeability of the mitochondrial membrane and cause apoptotic effectors to leak out. There is also a growing body of evidence indicating that nitric oxide is able to induce apoptosis by helping to dissipate the membrane potential of mitochondria and therefore make it more permeable.

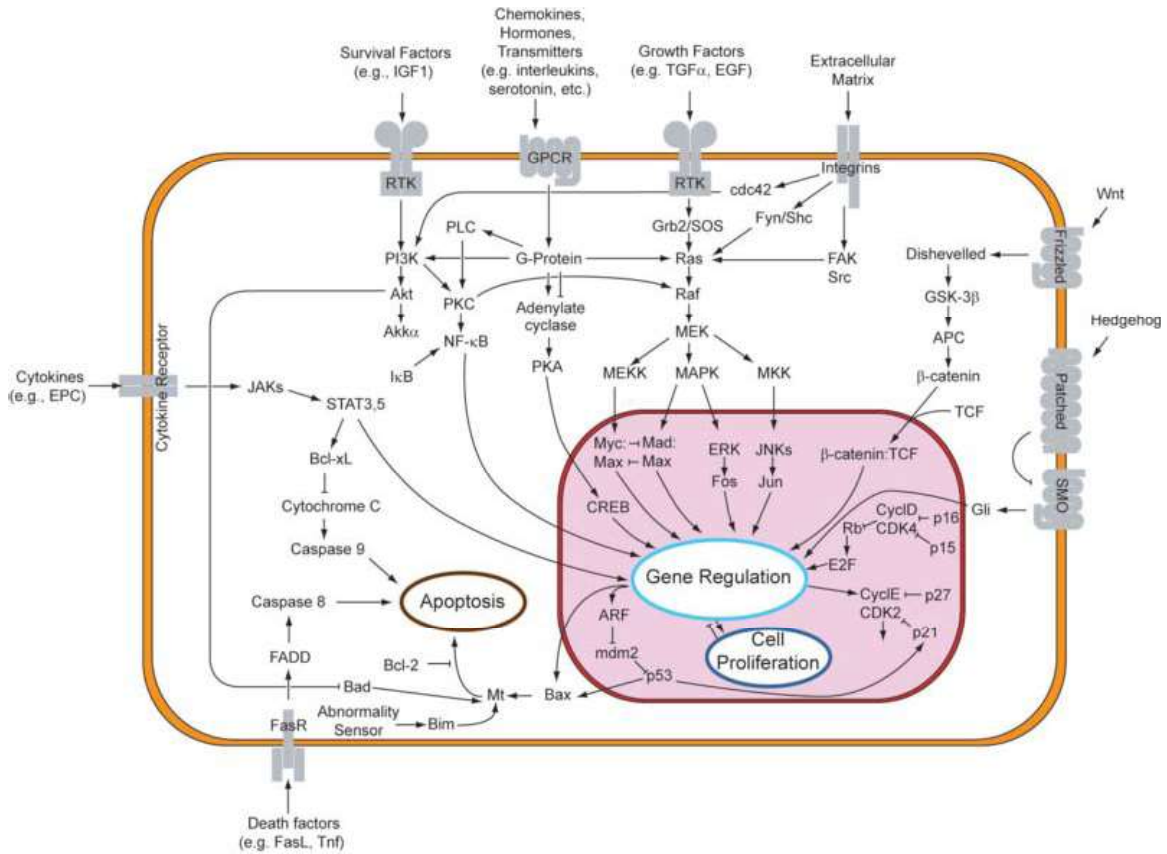
Mitochondrial proteins known as SMACs (second mitochondria-derived activator of caspases) are released into the cytosol following an increase in permeability. SMAC binds to *inhibitor of apoptosis proteins* (IAPs) and deactivates them, preventing the IAPs from arresting the apoptotic process and therefore allowing apoptosis to proceed. IAP also normally suppresses the activity of a group of cysteine proteases called caspases, which carry out the degradation of the cell, therefore the actual degradation enzymes can be seen to be indirectly regulated by mitochondrial permeability.

Cytochrome c is also released from mitochondria due to formation of a channel, MAC, in the outer mitochondrial membrane, and serves a regulatory function as it precedes morphological change associated with apoptosis. Once cytochrome c is released it binds with Apoptotic protease activating factor - 1 (*Apaf-1*) and ATP, which then bind to *pro-caspase-9* to create a protein complex known as an apoptosome. The apoptosome cleaves the pro-caspase to its active form of caspase-9, which in turn activates the effector *caspase-3*.

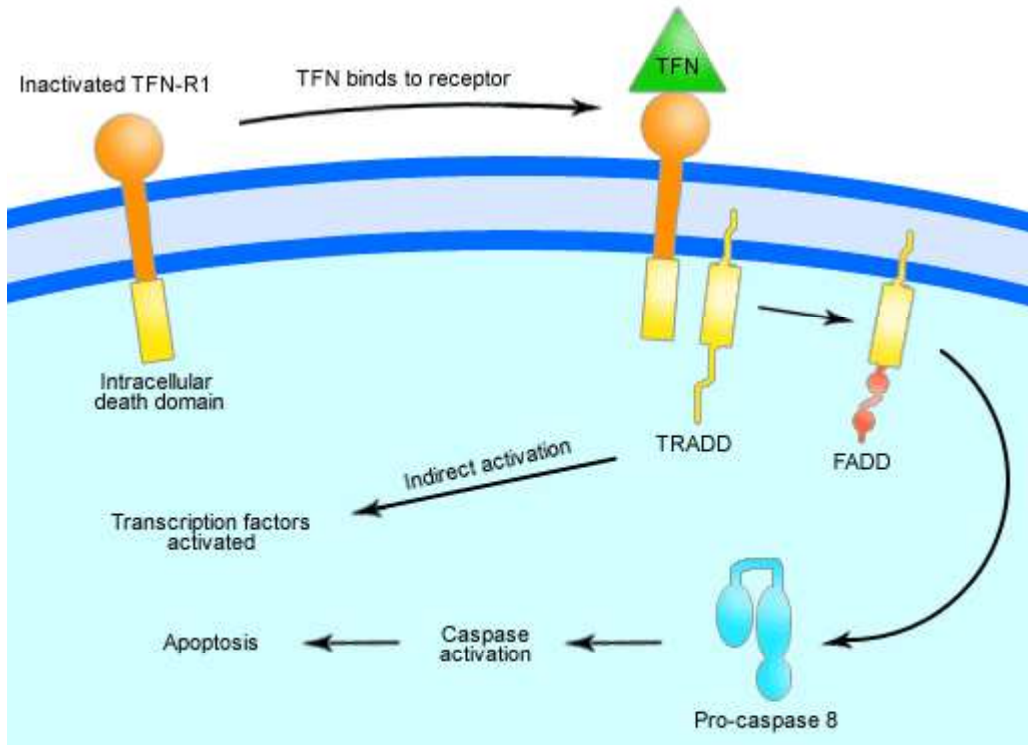
MAC, also called "Mitochondrial Outer Membrane Permeabilization Pore" is regulated by various proteins, such as those encoded by the mammalian *Bcl-2* family of anti-apoptotic genes, the homologs of the *ced-9* gene found in *C. elegans*. *Bcl-2* proteins are

able to promote or inhibit apoptosis by direct action on MAC/MOMP. Bax and/or Bak form the pore, while Bcl-2, Bcl-xL or Mcl-1 inhibit its formation.

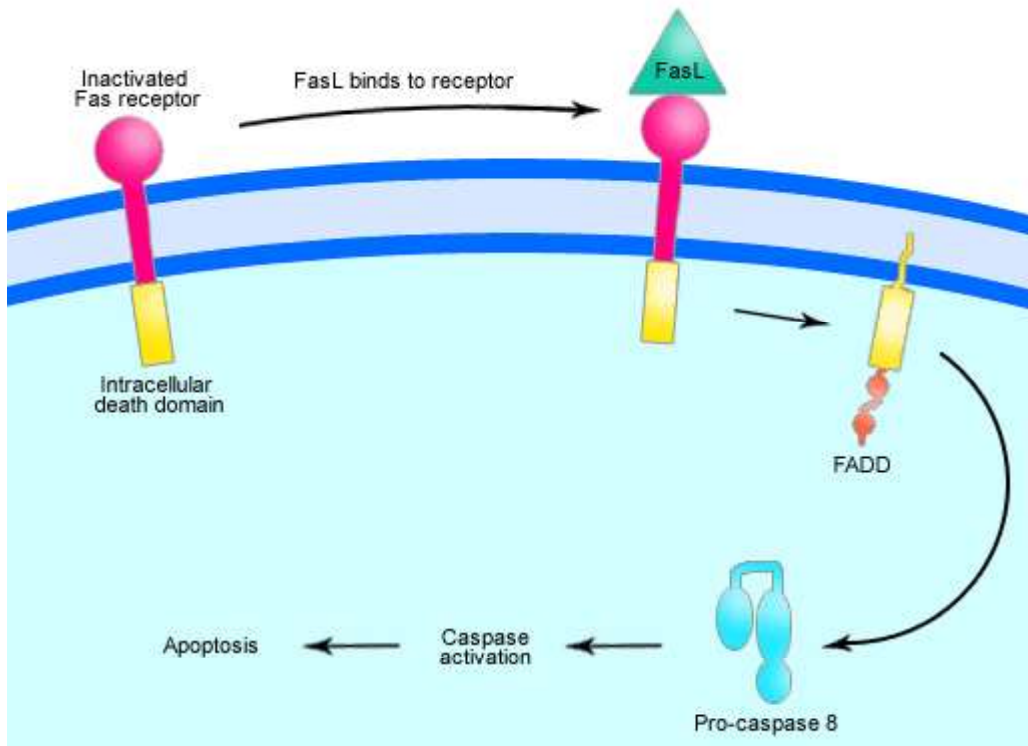
## Direct signal transduction



Overview of signal transduction pathways.



Overview of TNF signalling in apoptosis, an example of direct signal transduction



Overview of Fas signalling in apoptosis, an example of direct signal transduction

Two theories of the direct initiation of apoptotic mechanisms in mammals have been suggested: the *TNF-induced* (tumour necrosis factor) model and the *Fas-Fas ligand-mediated* model, both involving receptors of the *TNF receptor* (TNFR) family coupled to extrinsic signals.

### **TNF path**

TNF is a cytokine produced mainly by activated macrophages, and is the major extrinsic mediator of binary hipaloptic apoptosis. Most cells in the human body have two receptors for TNF: *TNF-R1* and *TNF-R2*. The binding of TNF to *TNF-R1* has been shown to initiate the pathway that leads to caspase activation via the intermediate membrane proteins *TNF receptor-associated death domain* (TRADD) and *Fas-associated death domain protein* (FADD). Binding of this receptor can also indirectly lead to the activation of transcription factors involved in cell survival and inflammatory responses. The link between TNF and apoptosis shows why an abnormal production of TNF plays a fundamental role in several human diseases, especially in autoimmune diseases.

### **Fas path**

The Fas receptor (also known as *Apo-1* or *CD95*) binds the Fas ligand (FasL), a transmembrane protein part of the TNF family. The interaction between Fas and FasL results in the formation of the *death-inducing signaling complex* (DISC), which contains the FADD, caspase-8 and caspase-10. In some types of cells (type I), processed caspase-8 directly activates other members of the caspase family, and triggers the execution of apoptosis of the cell. In other types of cells (type II), the *Fas-DISC* starts a feedback loop that spirals into increasing release of pro-apoptotic factors from mitochondria and the amplified activation of caspase-8.

### **Common components**

Following *TNF-R1* and *Fas* activation in mammalian cells a balance between pro-apoptotic (BAX, BID, BAK, or BAD) and anti-apoptotic (*Bcl-Xl* and *Bcl-2*) members of the *Bcl-2* family is established. This balance is the proportion of pro-apoptotic homodimers that form in the outer-membrane of the mitochondrion. The pro-apoptotic homodimers are required to make the mitochondrial membrane permeable for the release of caspase activators such as cytochrome c and SMAC. Control of pro-apoptotic proteins under normal cell conditions of non-apoptotic cells is incompletely understood, but in general, Bax or Bak are activated by the activation of BH3-only proteins, part of the *Bcl-2* family.

### **Caspase-independent apoptotic pathway**

There also exists a caspase-independent apoptotic pathway that is mediated by AIF (apoptosis-inducing factor).

## Execution



Histologic cross section of embryonic foot of mouse (*Mus musculus*) in 15.5 day of its development. There are still cells between fingers. (Full development of mouse lasts 27 days.) (Compare this image with image of leg of mouse.)

Many pathways and signals lead to apoptosis, but there is only one mechanism that actually causes the death of a cell. After a cell receives stimulus, it undergoes organized degradation of cellular organelles by activated proteolytic caspases. A cell undergoing apoptosis shows a characteristic morphology:

1. Cell shrinkage and rounding are shown because of the breakdown of the proteinaceous cytoskeleton by caspases.
2. The cytoplasm appears dense, and the organelles appear tightly packed.

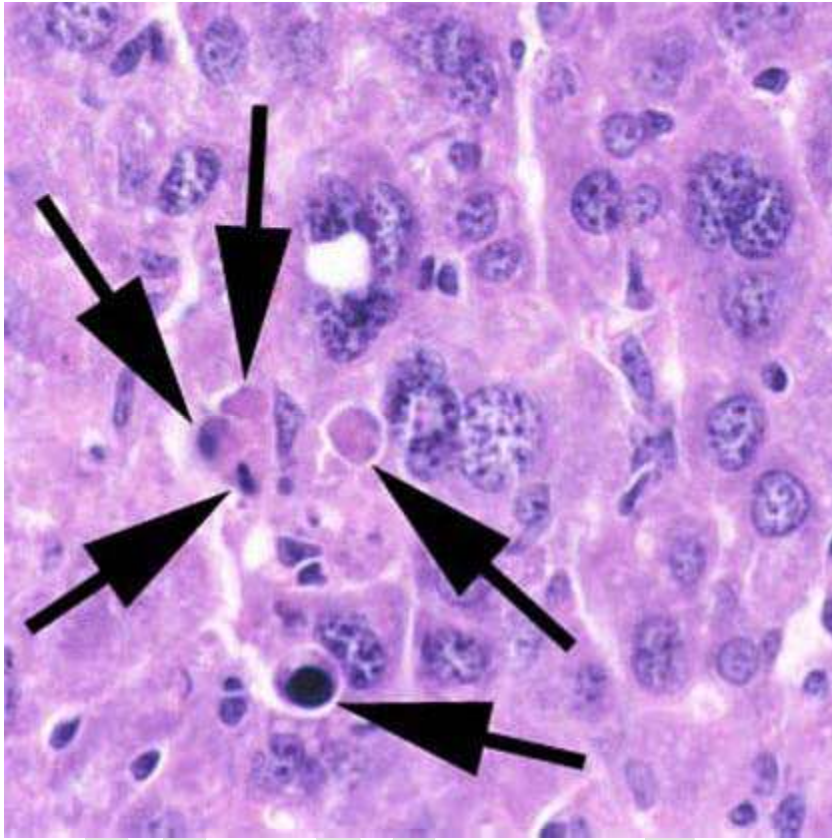
3. Chromatin undergoes condensation into compact patches against the nuclear envelope(also known as the perinuclear envelope) in a process known as pyknosis, a hallmark of apoptosis.
4. The nuclear envelope becomes discontinuous and the DNA inside it is fragmented in a process referred to as karyorrhexis. The nucleus breaks into several discrete *chromatin bodies* or *nucleosomal units* due to the degradation of DNA.
5. The cell membrane shows irregular buds known as blebs.
6. The cell breaks apart into several vesicles called *apoptotic bodies*, which are then phagocytosed.

Apoptosis progresses quickly and its products are quickly removed, making it difficult to detect or visualize. During karyorrhexis, endonuclease activation leaves short DNA fragments, regularly spaced in size. These give a characteristic "laddered" appearance on agar gel after electrophoresis. Tests for DNA laddering differentiate apoptosis from ischemic or toxic cell death.

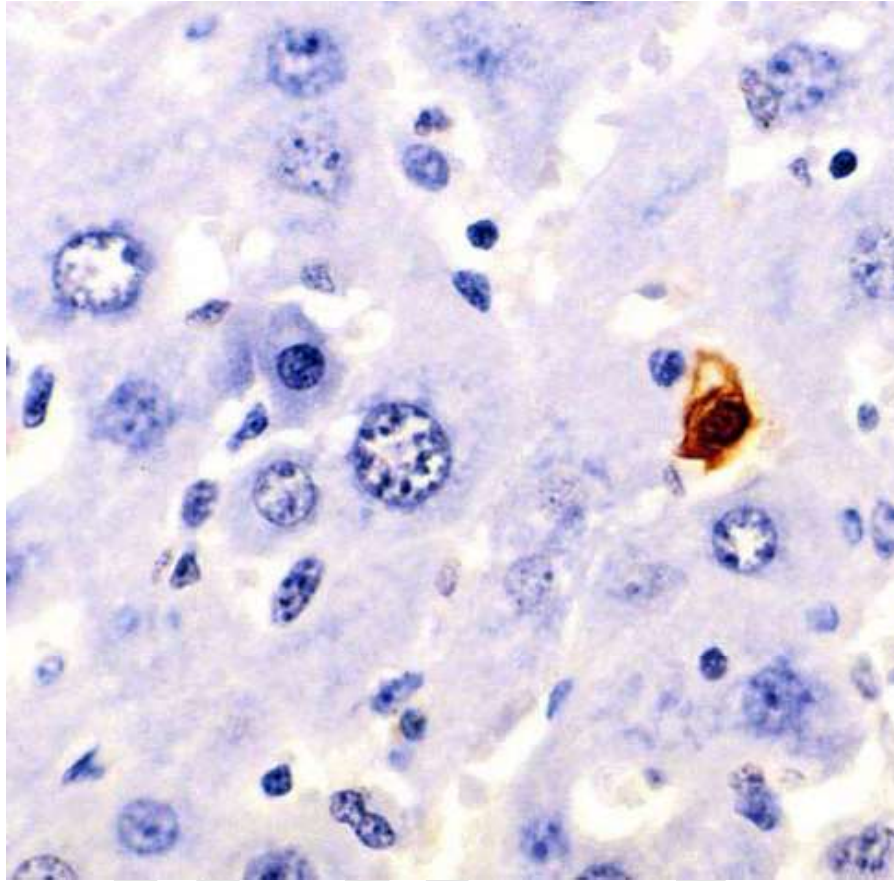
### **Removal of dead cells**

The removal of dead cells by neighboring phagocytic cells has been termed efferocytosis. Dying cells that undergo the final stages of apoptosis display phagocytotic molecules, such as phosphatidylserine, on their cell surface. Phosphatidylserine is normally found on the cytosolic surface of the plasma membrane, but is redistributed during apoptosis to the extracellular surface by a hypothetical protein known as scramblase. These molecules mark the cell for phagocytosis by cells possessing the appropriate receptors, such as macrophages. Upon recognition, the phagocyte reorganizes its cytoskeleton for engulfment of the cell. The removal of dying cells by phagocytes occurs in an orderly manner without eliciting an inflammatory response.

***Implication in disease***



A section of mouse liver showing several apoptotic cells, indicated by arrows



A section of mouse liver stained to show cells undergoing apoptosis (orange)

### **Defective apoptotic pathways**

The many different types of apoptotic pathways contain a multitude of different biochemical components, many of them not yet understood. As a pathway is more or less sequential in nature, it is a victim of causality; removing or modifying one component leads to an effect in another. In a living organism this can have disastrous effects, often in the form of disease or disorder. A discussion of every disease caused by modification of the various apoptotic pathways would be impractical, but the concept overlying each one is the same: the normal functioning of the pathway has been disrupted in such a way as to impair the ability of the cell to undergo normal apoptosis. This results in a cell that lives past its "use-by-date" and is able to replicate and pass on any faulty machinery to its progeny, increasing the likelihood of the cell becoming cancerous or diseased.

A recently-described example of this concept in action can be seen in the development of a lung cancer called NCI-H460. The *X-linked inhibitor of apoptosis protein* (XIAP) is overexpressed in cells of the H460 cell line. XIAPs bind to the processed form of caspase-9, and suppress the activity of apoptotic activator cytochrome c, therefore overexpression leads to a decrease in the amount of pro-apoptotic agonists. As a consequence, the balance of anti-apoptotic and pro-apoptotic effectors is upset in favour of the former, and the damaged cells continue to replicate despite being directed to die.

## Dysregulation of p53

The tumor-suppressor protein p53 accumulates when DNA is damaged due to a chain of biochemical factors. Part of this pathway includes alpha-interferon and beta-interferon, which induce transcription of the *p53* gene and result in the increase of p53 protein level and enhancement of cancer cell-apoptosis. p53 prevents the cell from replicating by stopping the cell cycle at G1, or interphase, to give the cell time to repair, however it will induce apoptosis if damage is extensive and repair efforts fail. Any disruption to the regulation of the *p53* or interferon genes will result in impaired apoptosis and the possible formation of tumors.

## HIV progression

The progression of the human immunodeficiency virus infection to AIDS is primarily due to the depletion of CD4+ T-helper lymphocytes, which leads to a compromised immune system. One of the mechanisms by which T-helper cells are depleted is apoptosis, which results from a series of biochemical pathways:

1. HIV enzymes deactivate anti-apoptotic *Bcl-2*. This does not directly cause cell death, but primes the cell for apoptosis should the appropriate signal be received. In parallel, these enzymes activate pro-apoptotic *procaspase-8*, which does directly activate the mitochondrial events of apoptosis.
2. HIV may increase the level of cellular proteins which prompt Fas-mediated apoptosis.
3. HIV proteins decrease the amount of CD4 glycoprotein marker present on the cell membrane.
4. Released viral particles and proteins present in extracellular fluid are able to induce apoptosis in nearby "bystander" T helper cells.
5. HIV decreases the production of molecules involved in marking the cell for apoptosis, giving the virus time to replicate and continue releasing apoptotic agents and virions into the surrounding tissue.
6. The infected CD4+ cell may also receive the death signal from a cytotoxic T cell.

Cells may also die as a direct consequence of viral infection. HIV-1 expression induces tubular cell G2/M arrest and apoptosis.

## Viral infection

Viruses can trigger apoptosis of infected cells via a range of mechanisms including:

- Receptor binding.
- Activation of protein kinase R (PKR).
- Interaction with p53.
- Expression of viral proteins coupled to MHC proteins on the surface of the infected cell, allowing recognition by cells of the immune system (such as Natural

Killer and cytotoxic T cells) that then induce the infected cell to undergo apoptosis.

Most viruses encode proteins that can inhibit apoptosis. Several viruses encode viral homologs of Bcl-2. These homologs can inhibit pro-apoptotic proteins such as BAX and BAK, which are essential for the activation of apoptosis. Examples of viral Bcl-2 proteins include the Epstein-Barr virus BHRF1 protein and the adenovirus E1B 19K protein. Some viruses express caspase inhibitors that inhibit caspase activity and an example is the CrmA protein of cowpox viruses. Whilst a number of viruses can block the effects of TNF and Fas. For example the M-T2 protein of myxoma viruses can bind TNF preventing it from binding the TNF receptor and inducing a response. Furthermore, many viruses express p53 inhibitors that can bind p53 and inhibit its transcriptional transactivation activity. Consequently p53 cannot induce apoptosis since it cannot induce the expression of pro-apoptotic proteins. The adenovirus E1B-55K protein and the hepatitis B virus HBx protein are examples of viral proteins that can perform such a function.

Interestingly, viruses can remain intact from apoptosis particularly in the latter stages of infection. They can be exported in the *apoptotic bodies* that pinch off from the surface of the dying cell and the fact that they are engulfed by phagocytes prevents the initiation of a host response. This favours the spread of the virus.

### ***Apoptosis in plants***

Programmed cell death in plants has a number of molecular similarities to animal apoptosis, but it also has differences, notably the presence of a cell wall and the lack of an immune system which removes the pieces of the dead cell. Instead of an immune response, the dying cell synthesizes substances to break itself down and places them in a vacuole which ruptures as the cell dies. Whether this whole process resembles animal apoptosis closely enough to warrant using the name *apoptosis* (as opposed to the more general *programmed cell death*) is unclear.

### ***Caspase Independent Apoptosis***

There is an extrinsic pathway that has been noticed in several toxicity studies. It was shown that an increase in calcium concentration within a cell, caused by drug activity, also has the ability to cause apoptosis via a calcium-binding calpain protease.

### ***Apoptosis Protein Subcellular Location Prediction***

In 2003, a method was developed for predicting subcellular location of apoptosis proteins. Subsequently, various different modes of Chou's pseudo amino acid composition were developed for improving the quality of predicting subcellular localization of apoptosis proteins based on their sequence information alone.

## Chapter 5

# Cytokine

**Cytokines** (Greek *cyto-*, cell; and *-kinos*, movement) are small cell-signaling protein molecules that are secreted by the glial cells of the nervous system and by numerous cells of the immune system and are a category of signaling molecules used extensively in intercellular communication. Cytokines can be classified as proteins, peptides, or glycoproteins; the term "cytokine" encompasses a large and diverse family of regulators produced throughout the body by cells of diverse embryological origin.

The term "cytokine" has been used to refer to the immunomodulating agents, such as interleukins and interferons. Biochemists disagree as to which molecules should be termed cytokines and which hormones. As we learn more about each, anatomic and structural distinctions between the two are fading. Classic protein hormones circulate in nanomolar ( $10^{-9}$ ) concentrations that usually vary by less than one order of magnitude. In contrast, some cytokines (such as IL-6) circulate in picomolar ( $10^{-12}$ ) concentrations that can increase up to 1,000-fold during trauma or infection. The widespread distribution of cellular sources for cytokines may be a feature that differentiates them from hormones. Virtually all nucleated cells, but especially endo/epithelial cells and resident macrophages (many near the interface with the external environment) are potent producers of IL-1, IL-6, and TNF- $\alpha$ . In contrast, classic hormones, such as insulin, are secreted from discrete glands (e.g., the pancreas). As of 2008, the current terminology refers to cytokines as immunomodulating agents. However, more research is needed in this area of defining cytokines and hormones.

Part of the difficulty with distinguishing cytokines from hormones is that some of the immunomodulating effects of cytokines are systemic rather than local. For instance, to use hormone terminology, the action of cytokines may be autocrine or paracrine in chemotaxis and endocrine as a pyrogen. Further, as molecules, cytokines are not limited to their immunomodulatory role. For instance, cytokines are also involved in several developmental processes during embryogenesis

## **Effects**

Each cytokine has a matching cell-surface receptor. Subsequent cascades of intracellular signalling then alter cell functions. This may include the upregulation and/or downregulation of several genes and their transcription factors, resulting in the production of other cytokines, an increase in the number of surface receptors for other molecules, or the suppression of their own effect by feedback inhibition.

The effect of a particular cytokine on a given cell depends on the cytokine, its extracellular abundance, the presence and abundance of the complementary receptor on the cell surface, and downstream signals activated by receptor binding; these last two factors can vary by cell type. Cytokines are characterized by considerable "redundancy", in that many cytokines appear to share similar functions.

It seems to be a paradox that cytokines binding to antibodies have a stronger immune effect than the cytokine alone. This may lead to lower therapeutic doses.

Said et al. showed that inflammatory cytokines cause an IL-10-dependent inhibition of CD4 T-cell expansion and function by up-regulating PD-1 levels on monocytes which leads to IL-10 production by monocytes after binding of PD-1 by PD-L.

## **Nomenclature**

Cytokines have been classed as lymphokines, interleukins, and chemokines, based on their presumed function, cell of secretion, or target of action. Because cytokines are characterised by considerable redundancy and pleiotropism, such distinctions, allowing for exceptions, are obsolete.

- The term *interleukin* was initially used by researchers for those cytokines whose presumed targets are principally leukocytes. It is now used largely for designation of newer cytokine molecules discovered every day and bears little relation to their presumed function. The vast majority of these are produced by T-helper cells.
- The term *chemokine* refers to a specific class of cytokines that mediates chemoattraction (chemotaxis) between cells.

## **Classification**

### **Structural**

Structural homology has been able to partially distinguish between cytokines that do not demonstrate a considerable degree of redundancy so that they can be classified into four types:

- The four- $\alpha$ -helix bundle family - Member cytokines have three-dimensional structures with four bundles of  $\alpha$ -helices. This family, in turn, is divided into three sub-families:

1. the IL-2 subfamily
2. the interferon (IFN) subfamily
3. the IL-10 subfamily.
  - The first of these three subfamilies is the largest. It contains several non-immunological cytokines including erythropoietin (EPO) and thrombopoietin (TPO). Also, four  $\alpha$ -helix bundle cytokines can be grouped into *long-chain* and *short-chain* cytokines.
- the IL-1 family, which primarily includes IL-1 and IL-18
- the IL-17 family, which has yet to be completely characterized, though member cytokines have a specific effect in promoting proliferation of T-cells that cause cytotoxic effects

## Functional

A classification that proves more useful in clinical and experimental practice divides immunological cytokines into those that enhance cellular immune responses, type 1 (IFN- $\gamma$ , TGF- $\beta$ , etc.), and type 2 (IL-4, IL-10, IL-13, etc.), which favor antibody responses.

A key focus of interest has been that cytokines in one of these two sub-sets tend to inhibit the effects of those in the other. Dysregulation of this tendency is under intensive study for its possible role in the pathogenesis of autoimmune disorders.

Several inflammatory cytokines are induced by oxidant stress. The fact that cytokines themselves trigger the release of other cytokines and also lead to increased oxidant stress makes them important in chronic inflammation.

## Cytokine receptors

In recent years, the cytokine receptors have come to demand the attention of more investigators than cytokines themselves, partly because of their remarkable characteristics, and partly because a deficiency of cytokine receptors has now been directly linked to certain debilitating immunodeficiency states. In this regard, and also because the redundancy and pleiomorphism of cytokines are, in fact, a consequence of their homologous receptors, many authorities think that a classification of cytokine receptors would be more clinically and experimentally useful.

A classification of cytokine receptors based on their three-dimensional structure has, therefore, been attempted. Such a classification, though seemingly cumbersome, provides several unique perspectives for attractive pharmacotherapeutic targets.

- Immunoglobulin (Ig) superfamily, which are ubiquitously present throughout several cells and tissues of the vertebrate body, and share structural homology with immunoglobulins (antibodies), cell adhesion molecules, and even some cytokines. Examples: IL-1 receptor types.
- Haemopoietic Growth Factor (type 1) family, whose members have certain conserved motifs in their extracellular amino-acid domain. The IL-2 receptor

belongs to this chain, whose  $\gamma$ -chain (common to several other cytokines) deficiency is directly responsible for the x-linked form of Severe Combined Immunodeficiency (X-SCID).

- Interferon (type 2) family, whose members are receptors for IFN  $\beta$  and  $\gamma$ .
- Tumor necrosis factors (TNF) (type 3) family, whose members share a cysteine-rich common extracellular binding domain, and includes several other non-cytokine ligands like CD40, CD27 and CD30, besides the ligands on which the family is named (TNF).
- Seven transmembrane helix family, the ubiquitous receptor type of the animal kingdom. All G protein-coupled receptors (for hormones and neurotransmitters) belong to this family. Chemokine receptors, two of which act as binding proteins for HIV (CXCR4 and CCR5), also belong to this family.

## ***Disease***

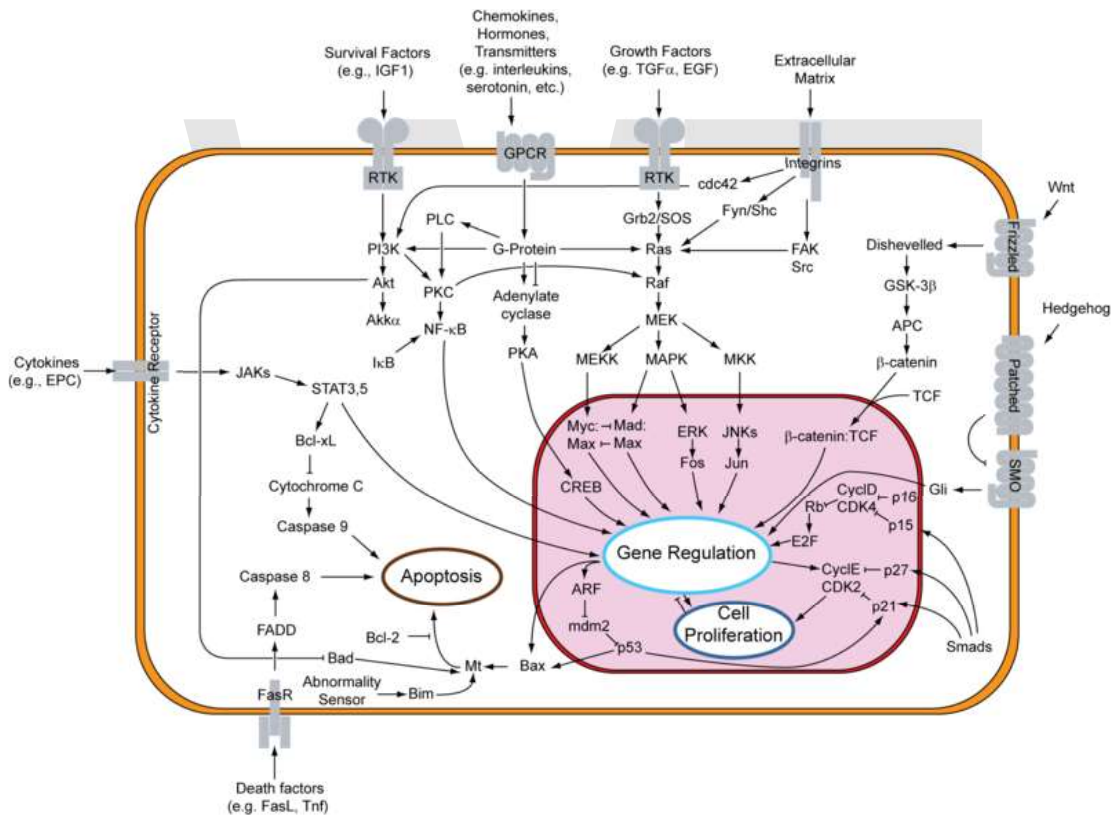
Adverse effects of cytokines have been linked to many disease states and conditions ranging from major depression and Alzheimer's disease to cancer with levels either being elevated or changed. Oversecretion of cytokines can trigger a dangerous syndrome known as a cytokine storm; this may have been the cause of severe adverse events during a clinical trial of TGN1412.

## **Plasma levels**

Plasma levels of various cytokines may give information on the presence, or even predictive value of inflammatory processes involved in autoimmune diseases such as rheumatoid arthritis, as well as immunomodulatory effects of foods or drugs . In addition, elevated levels of IL-7, an important cytokine involved in T cell homeostasis, have been detected in the plasma of HIV-infected patients.

## Chapter 6

# Signal Transduction



Overview of signal transduction pathways

Signal transduction comes from the verb to 'transduce' meaning to 'lead across'. In biology **signal transduction** is the process by which an extracellular signaling molecule activates a membrane receptor that in turn alters intracellular molecules to create a response. Transmembrane receptors span the cell membrane, with part of the receptor outside and part inside the cell. The chemical signal binds to the outer portion of the receptor, changing its shape and conveying another signal inside the cell. Some chemical

messengers, such as testosterone, can pass through the cell membrane, and bind directly to receptors in the cytoplasm or nucleus.

Sometimes there is a cascade of signals within the cell. With each step of the cascade, the signal can be amplified, so a small signal can result in a large response. Eventually, the signal creates a change in the cell, either in the expression of the DNA in the nucleus or in the activity of enzymes in the cytoplasm.

These processes can take milliseconds (for ion flux), minutes (for protein- and lipid-mediated kinase cascades), hours, or days (for gene expression).

## ***Signaling molecules***

Most signal transduction involves the binding of extracellular signaling molecules (and ligands) to cell-surface receptors. While triggering events inside the cell, such receptors typically face outward from the plasma membrane. Intracellular signaling cascades can also be triggered through cell-substratum interactions. One example is integrins, which bind ligands found within the extracellular matrix. Steroids are another example of extracellular signaling molecules that may cross the plasma membrane due to their lipophilic or hydrophobic nature. Many, but not all, steroid hormones have receptors within the cytoplasm, and usually act by stimulating the binding of their receptors to the promoter region of steroid-responsive genes. Within multicellular organisms, numerous small molecules and polypeptides serve to coordinate a cell's individual biological activity within the context of the organism as a whole. These molecules have been functionally classified as:

- hormones (e.g., melatonin),
- growth factors (e.g. epidermal growth factor),
- extra-cellular matrix components (e.g., fibronectin),
- cytokines (e.g., interferon-gamma),
- chemokines (e.g., RANTES),
- neurotransmitters (e.g., acetylcholine), and
- neurotrophins (e.g., nerve growth factor).
- active oxygen species and other electronically-activated compounds.

Most of these classifications do not take into account the molecular nature of each class member. For example, as a class, neurotransmitters consist of neuropeptides such as endorphins and small molecules such as serotonin and dopamine. Hormones, another generic class of molecules capable of initiating signal transduction, include insulin (a polypeptide), testosterone (a steroid), and epinephrine (an amino acid derivative, in essence a small organic molecule).

The classification of one molecule into one class or another is not exact. For example, epinephrine and norepinephrine, secreted by the central nervous system, act as neurotransmitters. However, when secreted by the adrenal medulla, epinephrine acts as a hormone.

## ***Environmental stimuli***

In bacteria and other single-cell organisms, the variety of signal transduction processes possible influences how it reacts and responds to its environment. In multicellular organisms, numerous signal transduction processes are required for coordinating the behavior of individual cells to support the function of the organism as a whole. The complexity of an organism's signal transduction processes tends to increase with the complexity of the organism itself. Sensing of both the external and internal environments at the cellular level relies on signal transduction. Many disease processes, such as diabetes, heart disease, autoimmunity, and cancer arise from defects in signal transduction pathways, further highlighting the critical importance of signal transduction to biology, as well as medicine.

Various environmental stimuli, in addition to many of the regular signal transduction stimuli listed above, initiate signal transmission processes in complex organisms. Environmental stimuli may also be molecular in nature (as above) or more physical, such as light striking cells in the retina of the eye, odorants binding to odorant receptors in the nasal epithelium, and bitter and sweet tastes stimulating taste receptors in the taste buds.

Certain microbial molecules, e.g., viral nucleotides, bacterial lipopolysaccharides, and protein antigens, are able to elicit an immune system response against invading pathogens, mediated by signal transduction processes. An immune response may occur independent of signal transduction stimulation by other molecules, as is the case for signal transduction by way of the Toll-like receptor or with help from stimulatory molecules located at the cell surface of other cells, as is the case for T-cell receptor signaling.

Unicellular organisms may also respond to environmental stimuli through the activation of signal transduction pathways. For example, slime molds secrete cyclic-AMP upon starvation, which stimulates individual cells in the immediate environment to aggregate. Yeast use mating factors to determine the mating types of other yeast and to participate in sexual reproduction.

## ***Cellular responses***

Activation of genes, alterations in metabolism, the continued proliferation and death of the cell, and the stimulation or suppression of locomotion, are some of the cellular responses to extracellular stimulation that require signal transduction. Gene activation leads to further cellular effects, since the protein products of many of the responding genes include enzymes and transcription factors themselves. Transcription factors produced as a result of a signal transduction cascade can, in turn, activate yet more genes. Therefore an initial stimulus can trigger the expression of an entire cohort of genes, and this, in turn, can lead to the activation of any number of complex physiological events. These events include the increased uptake of glucose from the blood stream stimulated by insulin and the migration of neutrophils to sites of infection

stimulated by bacterial products. The set of genes and the order in which they are activated in response to stimuli are often referred to as a *genetic program*.

Neurotransmitters are ligands that are capable of binding to ion channel proteins, resulting in their opening to allow the rapid flow of a particular ion across the plasma membrane. This results in an altering of the cell's membrane potential and is important for processes such as the neural conduction of electrochemical impulses. Ligands can be freely soluble, or can be found on the surface of other cells or within the extracellular matrix. Such cell surface or extracellular matrix ligands signal between cells when they come in contact with each other, such as when a phagocytic cell presents antigens to lymphocytes, or upon adhesion to the extracellular matrix, as when integrins at the cell surface of fibroblasts engage fibronectin.

Most mammalian cells require stimulation to control not only cell division but also survival. In the absence of growth factor stimulation, programmed cell death ensues in most cells. Such requirements for extra-cellular stimulation are necessary for controlling cell behavior in the context of both unicellular and multi-cellular organisms. Signal transduction pathways are perceived to be so central to biological processes that it is not surprising that a large number of diseases have been attributed to their dysregulation.

Discussed below are how signal transduction via various classes of receptor may lead to the above cellular responses.

### ***Types of receptor***

Receptors can be roughly divided into two major classes:

1. Intracellular receptors and
2. Cell-surface receptors.

Ligand-gated ion channel receptors are a class of receptor that may occur both at the cell-surface or intracellularly.

Solely intracellular receptors include those for steroid hormones, thyroid hormone, retinoic acid, and derivatives of vitamin D<sub>3</sub>. In contrast to ligands that bind to cell surface receptors to initiate signal transduction, these ligands must cross the cell membrane.

Major categories of intracellular receptors include G-protein linked receptors and Tyrosine Kinase receptors.

### **Cell-surface receptors**

Cell-surface receptors are integral transmembrane proteins and recognize the vast majority of extracellular signaling molecules. Transmembrane receptors span the plasma membrane of the cell, with one part of the receptor on the outside of the cell (the

*extracellular domain*), and the other on the inside of the cell (the *intracellular domain*). Signal transduction occurs as a result of stimulatory molecule or the binding of a ligand to its extracellular domain; the ligand itself does not pass through the plasma membrane prior to receptor-binding.

Binding of a ligand to a cell-surface receptor stimulates a series of events inside the cell, with different types of receptor stimulating different intracellular responses. Receptors typically respond to only the binding of a specific ligand. Upon binding, the ligand initiates the transmission of a signal across the plasma membrane by inducing a change in the shape or conformation of the intracellular part of the receptor. Often, such changes in conformation either result in the activation of an enzymatic activity contained within the receptor or expose a binding site for other signaling proteins within the cell. Once these proteins bind to the receptor, they themselves may become active and propagate the signal into the cytoplasm.

In eukaryotic cells, most intracellular proteins activated by a ligand/receptor interaction possess an enzymatic activity. These enzymes include tyrosine kinase, heterotrimeric G proteins, small GTPases, various serine/threonine protein kinases, phosphatases, lipid kinases, and hydrolases. Some receptor-stimulated enzymes create specific second messengers including cyclic nucleotides, such as cyclic AMP (cAMP) and cyclic GMP (cGMP), Phosphatidylinositol derivatives, such as Phosphatidylinositol-trisphosphate (PIP<sub>3</sub>), Diacylglycerol (DAG) and Inositol-triphosphate (IP<sub>3</sub>), IP<sub>3</sub>, controlling the release of intracellular calcium stores into the cytoplasm. Other activated proteins interact with adapter proteins. Adapter proteins facilitate interactions between other signaling proteins, and coordinate the formation of signaling complexes necessary to produce an appropriate cellular response to a particular stimulus. Enzymes and adapter proteins are both responsive to various second messenger molecules.

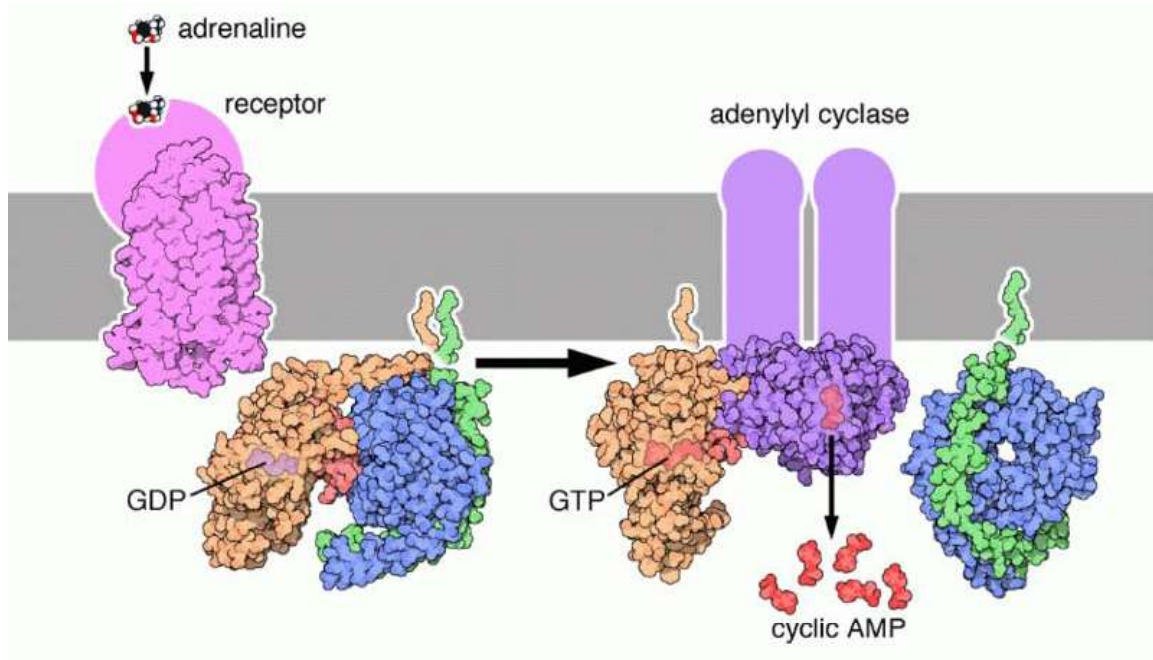
Many of the enzymes activated as part of the signal transduction mechanism and also many adapter proteins have been found to possess specialized protein domains that bind to specific secondary messenger molecules. For example, calcium ions bind specifically to the EF hand domains of calmodulin, allowing this molecule to bind and activate Calmodulin-dependent kinase. PIP<sub>3</sub>, PIP<sub>2</sub> and other phosphoinositides may bind to the Pleckstrin homology domains of proteins such as the kinase protein AKT again with activation activity.

There are many different classes of transmembrane receptor that recognize different extracellular signaling molecules. Specific example are:

1. G-protein coupled receptors, e.g., Chemokine receptors
2. Receptor tyrosine kinases, e.g., Growth factor receptors,
3. Integrins
4. Toll-like receptors

Further examples are given in the transmembrane receptor article.

## G-protein-coupled receptors



Signal transduction from a G-protein-linked receptor following interaction with its hormone ligand

G-protein-coupled receptors (GPCRs) are a family of integral membrane proteins that possess seven membrane-spanning domains, and are linked to a guanine nucleotide-binding protein (or heterotrimeric G protein). Many receptors make up this family, including adrenergic receptors, neurotransmitter receptors, olfactory receptors, opioid receptors, chemokine receptors, and rhodopsin.

Signal transduction by a GPCR begins with an inactive G protein coupled to the receptor. An inactive G protein exists as a heterotrimer, a molecule composed of three different protein subunits:  $G\alpha$ ,  $G\beta$ , and  $G\gamma$ . Once the GPCR recognizes a ligand, the shape (conformation) of the receptor changes to mechanically activate the G protein, and causes one subunit ( $G\alpha$ ) to bind a molecule of GTP (causing activation) and dissociate from the other two G-protein subunits ( $G\beta$  and  $G\gamma$ ). The dissociation exposes sites on the G-protein subunits that interact with other molecules. The activated G protein subunits detach from the receptor and initiate signaling from many *downstream* effector proteins. These include phosphodiesterases and adenylyl cyclases, phospholipases, and ion channels that permit the release of second messenger molecules such as cyclic-AMP (cAMP), cyclic-GMP (cGMP), inositol triphosphate (IP<sub>3</sub>), diacylglycerol (DAG), and calcium ( $Ca^{2+}$ ) ions. For example, a rhodopsin molecule in the plasma membrane of a retina cell in the eye that was activated by a photon can activate up to 2000 effector molecules (in this case, transducin) per second.

The total strength of signal amplification by a GPCR is determined by:

- *The lifetime of the ligand-receptor-complex.* If the ligand-receptor-complex is stable, it takes longer for the ligand to dissociate from its receptor, thus the receptor will remain active for longer and will activate more effector proteins.
- *The amount and lifetime of the receptor-effector protein-complex.* The more effector protein is available to be activated by the receptor, and the faster the activated effector protein can dissociate from the receptor, the more effector protein will be activated in the same amount of time.
- *Deactivation of the activated receptor.* A receptor that is engaged in a hormone-receptor-complex can be deactivated, either by covalent modification (for example, phosphorylation) or by internalization.
- *Deactivation of effectors through intrinsic enzymatic activity.* Either small or large G-proteins possess intrinsic GTPase activity, which controls the duration of the triggered signal. This activity may be increased through the action of other proteins such as GTPase-activating proteins (GAPs).

The idea that G-protein-coupled receptors, to be specific, chemokine receptors, participate in cancer development is suggested by a study wherein a point mutation was inserted into the gene encoding the chemokine receptor CXCR2. Cells transfected with the CXCR2 mutant underwent a malignant transformation. The result of the point mutation was the expression of CXCR2 in an active conformation, despite the absence of chemokine-binding (the CXCR2 mutant is said to be "constitutively active").

### **Receptor tyrosine kinases**

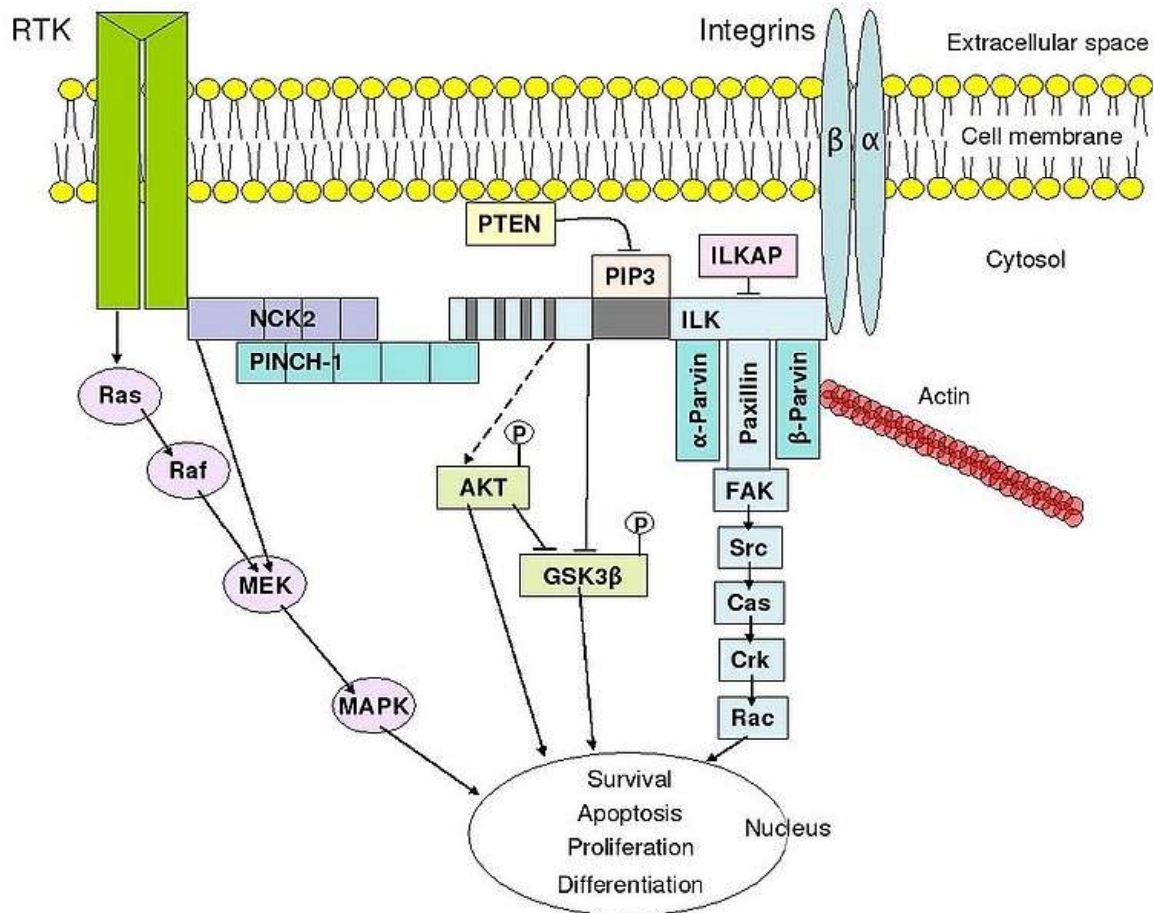
Receptor tyrosine kinases (RTKs) are transmembrane proteins with an intracellular kinase domain and an extracellular domain that binds ligand. There are many RTK proteins that are classified into subfamilies depending on their structural properties and ligand specificity. These include many growth factor receptors such as insulin receptor and the insulin-like growth factor receptors, and many others receptors. To conduct their biochemical signals, RTKs need to form dimers in the plasma membrane. The dimer is stabilized by ligand binding by the receptor. Interaction between the two cytoplasmic domains of the dimer is thought to stimulate autophosphorylation of tyrosines within the cytoplasmic tyrosine kinase domains of the RTKs causing their conformational changes. The kinase domain of the receptors is subsequently activated, initiating signaling cascades of phosphorylation of downstream cytoplasmic molecules. These signals are essential to various cellular processes, such as control of cell growth, differentiation, metabolism, and migration.

As is the case with G-Protein-coupled receptors, proteins that bind GTP play a major role in transmission of signal from the activated RTK into the cell. In this case, the G proteins are members of the Ras, Rho, and Raf families, referred to collectively as small G proteins. These proteins act as molecular switches that are usually tethered to membranes by isoprenyl groups linked to their carboxyl ends. Thus, upon activation, they are responsible for the recruitment of proteins to specific membrane subdomains where they participate in signaling. Activated RTKs, in turn, activate small G proteins, which in turn activate Guanine Nucleotide Exchange Factors, such as SOS1. Once activated, these

exchange factors can activate many more small G-proteins, thus amplifying the receptors initial signal.

As with the mutation of G-protein coupled receptors, the mutation of certain RTK genes can result in the expression of receptors that exist in a constitutively-activate state. Such mutated RTK genes may act as oncogenes, genes that contribute to the initiation or progression of cancer.

### Integrins



An overview of integrin-mediated signal transduction, adapted from Hehlgens *et al.* (2007).

Integrins are produced by a wide variety of cell types, and play a role in the attachment of a cell to the extracellular matrix (ECM) and to other cells, and in the signal transduction of signals received from extracellular matrix components such as fibronectin, collagen, and laminin. Ligand-binding to the extracellular domain of integrins induces a conformational change within the protein and a clustering of the protein at the cell surface to initiate signal transduction. Integrins lack kinase activity, and integrin-mediated signal transduction is achieved through a variety of intracellular protein kinases

and adaptor molecules such as integrin-linked kinase (ILK), focal-adhesion kinase (FAK), talin, paxillin, parvins, p130Cas, Src-family kinases, and GTPases of the Rho family, the main protein coordinating signal transduction being ILK. As shown in the overview to the right, cooperative integrin and receptor tyrosine kinase signaling determine cellular survival, apoptosis, proliferation, and differentiation.

Important differences exist between integrin-signaling in circulating blood cells and that in non-circulating blood cells such as epithelial cells. Integrins at the cell-surface of circulating cells are inactive under normal physiological conditions. For example, cell-surface integrins on circulating leukocytes are maintained in an inactive state to avoid epithelial cell attachment. Only in response to appropriate stimuli are leukocyte integrins converted into an active form, such as those received at the site of an inflammatory response. In a similar manner, it is important that integrins at the cell surface of circulating platelets are kept in an inactive state under normal conditions to avoid thrombosis. Epithelial cells, in contrast, have active integrins at their cell surface under normal conditions, which help maintain their stable adhesion to underlying stromal cells, which provide appropriate signals to maintain their survival and differentiation.

### **Toll-like receptors**

When activated, Toll-like receptors (TLRs) recruit adapter molecules within the cytoplasm of cells in order to propagate a signal. Four adapter molecules are known to be involved in signaling. These proteins are known as MyD88, Tirap (also called Mal), Trif, and Tram. The adapters activate other molecules within the cell, including certain protein kinases (IRAK1, IRAK4, TBK1, and IKKi) that amplify the signal, and ultimately lead to the induction or suppression of genes that orchestrate the inflammatory response. In all, thousands of genes are activated by TLR signaling, and, together, the TLRs constitute one of the most powerful and important gateways for gene modulation.

### **Ligand-gated ion channel receptors**

*A ligand-activated ion channel* will recognize its ligand, and then undergo a structural change that opens a gap (channel) in the plasma membrane through which ions can pass. These ions will then relay the signal. An example for this mechanism is found in the receiving cell, or post-synaptic cell of a neural synapse.

By contrast, other ion channels open in response to a change in cell potential, that is, the difference of the electrical charge across the membrane. In neurons, this mechanism underlies the action potentials that travel along nerves. The influx of ions that occurs in response to ligand-gated ion channels often induce action potentials by depolarizing the membrane of the post-synaptic cells, which results in the wave-like opening of voltage-gated ion channels. In addition, calcium ions are also commonly allowed into the cell during ligand-induced ion channel opening. This calcium can act as a classical second messenger, setting in motion signal transduction cascades and altering the cellular physiology of the responding cell. This may result in strengthening of the synapse

between the pre- and post-synaptic cells by remodeling the dendritic spines involved in the synapse.

## Intracellular receptors

Intracellular receptors include nuclear receptors and cytoplasmic receptors, and are soluble proteins localized within the nucleoplasm or the cytoplasm, respectively. The typical ligands for nuclear receptors are lipophilic hormones, with steroid hormones (for example, testosterone, progesterone, and cortisol) and derivatives of vitamin A and D among them. To reach its receptor and initiate signal transduction, the hormone must pass through the plasma membrane, usually by passive diffusion. The nuclear receptors are ligand-activated transcription activators; on binding with the ligand (the hormone), the ligands will pass through the nuclear membrane into the nucleus and enable the transcription of a certain gene and, thus, the production of a protein.

The nuclear receptors that were activated by the hormones attach at the DNA at receptor-specific *Hormone-Responsive Elements (HREs)*, DNA sequences that are located in the promoter region of the genes that are activated by the hormone-receptor complex. As this enables the transcription of the according gene, these hormones are also called *inductors of gene expression*. The activation of gene transcription is much slower than signals that directly affect existing proteins. As a consequence, the effects of hormones that use nucleic receptors are usually long-term. Although the signal transduction via these soluble receptors involves only a few proteins, the details of gene regulation are yet not well understood. The nucleic receptors all have a similar, modular structure:

N-AAAABBBBCCCCDDDDDEEEEFFFF-C

where CCCC is the DNA-binding domain that contains zinc fingers, and EEEE the ligand-binding domain. The latter is also responsible for dimerization of most nuclear receptors prior to DNA binding. As a third function, it contains structural elements that are responsible for transactivation, used for communication with the translational apparatus. The zinc fingers in the DNA-binding domain stabilize DNA binding by holding contact to the phosphate backbone of the DNA. The DNA sequences that match the receptor are usually hexameric repeats, either normal, inverted, or everted. The sequences are quite similar, but their orientation and distance are the parameters by which the DNA-binding domains of the receptors can tell them apart.

**Steroid receptors** are a subclass of nuclear receptors, located primarily within the cytosol. In the absence of steroid hormone, the receptors cling together in a complex called an *aporeceptor complex*, which also contains chaperone proteins (also known as *heatshock proteins* or *Hsps*). The *Hsps* are necessary to activate the receptor by assisting the protein to fold in a way such that the signal sequence that enables its passage into the nucleus is accessible.

Steroid receptors can also have a *repressive* effect on gene expression, when their transactivation domain is hidden so it cannot activate transcription. Furthermore, steroid

receptor activity can be enhanced by phosphorylation of serine residues at their N-terminal end, as a result of another signal transduction pathway, for example, a by a growth factor. This behaviour is called *crosstalk*.

**RXR- and orphan-receptors** These nuclear receptors can be activated by

- a *classic* endocrine-synthesized hormone that entered the cell by diffusion
- a hormone that was built within the cell (for example, retinol) from a precursor or prohormone, which can be brought to the cell through the bloodstream
- a hormone that was completely synthesized within the cell, for example, prostaglandin.

These receptors are located in the nucleus and are *not* accompanied by *chaperone proteins*. In the absence of hormone, they bind to their specific DNA sequence, repressing the gene. Upon activation by the hormone, they activate the transcription of the gene that they were repressing.

Certain intracellular receptors of the immune system are examples of cytoplasmic receptors. Recently-identified NOD like receptors (NLRs) reside in the cytoplasm of specific eukaryotic cells and interact with particular ligands, such as microbial molecules, using a leucine-rich repeat (LRR) motif that is similar to the ligand-binding motif of the extracellular receptors known as TLRs. Some of these molecules (e.g., NOD1 and NOD2) interact with an enzyme called RICK kinase (or RIP2 kinase) that activates NF- $\kappa$ B signaling, whereas others (e.g., NALP3) interact with inflammatory caspases (e.g., caspase 1) and initiate processing of particular cytokines (e.g., interleukin-1 $\beta$ ). Similar receptors exist inside plant cells and are called Plant R Proteins. Another type of cytoplasmic receptor also has a role in immune surveillance. These receptors are known as RNA Helicases and include RIG-I, MDA5, and LGP2.

## **Second messengers**

Intracellular signal transduction is largely carried out by second messenger molecules.

### **Calcium**

$\text{Ca}^{2+}$  concentration is usually maintained at a very low level in the cytosol by sequestration in the smooth endoplasmic reticulum and the mitochondria.  $\text{Ca}^{2+}$  release from the endoplasmic reticulum into the cytosol results in the binding of the released  $\text{Ca}^{2+}$  to signaling proteins that are then activated. There are two combined receptor/ion channel proteins that perform the task of controlled transport of  $\text{Ca}^{2+}$ :

- The *InsP<sub>3</sub>-receptor* will transport  $\text{Ca}^{2+}$  upon interaction with inositol triphosphate (thus the name) on its cytosolic side. It consists of four identical subunits.
- The *ryanodine receptor* is named after the plant alkaloid ryanodine. It is similar to the  $\text{InsP}_3$  receptor and stimulated to transport  $\text{Ca}^{2+}$  into the cytosol by recognizing  $\text{Ca}^{2+}$  on its cytosolic side, thus establishing a feedback mechanism; a

small amount of  $\text{Ca}^{2+}$  in the cytosol near the receptor will cause it to release even more  $\text{Ca}^{2+}$ . It is especially important in neurons and muscle cells. In heart and pancreas cells, another second messenger (cyclic-ADP ribose) takes part in the receptor activation. The localized and time-limited activity of  $\text{Ca}^{2+}$  in the cytosol is also called a *Ca<sup>2+</sup> wave*. Once released into the cytosol from intracellular stores or extracellular sources,  $\text{Ca}^{2+}$  acts as a signal molecule within the cell. This works by tightly limiting the time and space when  $\text{Ca}^{2+}$  is free (and thus active). Therefore, the concentration of free  $\text{Ca}^{2+}$  within the cell is usually very low; it is stored within organelles, usually the endoplasmic reticulum (sarcoplasmic reticulum in muscle cells), where it is bound to molecules like calreticulin.

$\text{Ca}^{2+}$  is used in a multitude of processes, among them muscle contraction, release of neurotransmitter from nerve endings, vision in retina cells, proliferation, secretion, cytoskeleton management, cell migration, gene expression, and metabolism. The three main pathways that lead to  $\text{Ca}^{2+}$  activation are :

1. G protein-regulated pathways
2. Pathways regulated by receptor-tyrosine kinases
3. Ligand- or current-regulated ion channels

There are two different ways by which  $\text{Ca}^{2+}$  can regulate proteins:

1. A direct recognition of  $\text{Ca}^{2+}$  by the protein
2. Binding of  $\text{Ca}^{2+}$  in the active site of an enzyme.

One of the best-studied interactions of  $\text{Ca}^{2+}$  with a protein is the regulation of calmodulin by  $\text{Ca}^{2+}$ . Calmodulin itself can regulate other proteins, or be part of a larger protein (for example, phosphorylase kinase). The  $\text{Ca}^{2+}$ /calmodulin complex plays an important role in proliferation, mitosis, and neural signal transduction.

## **Lipophilic**

Lipophilic second messenger molecules are derived from lipids that normally reside in cellular membranes. Enzymes stimulated by activated receptors modify the lipids, converting them into second messengers.

Diacylglycerol is a lipophilic second messenger, required for the activation of protein kinase C. Ceramide, the eicosanoids, and lysophosphatidic acid are also lipophilic second messengers.

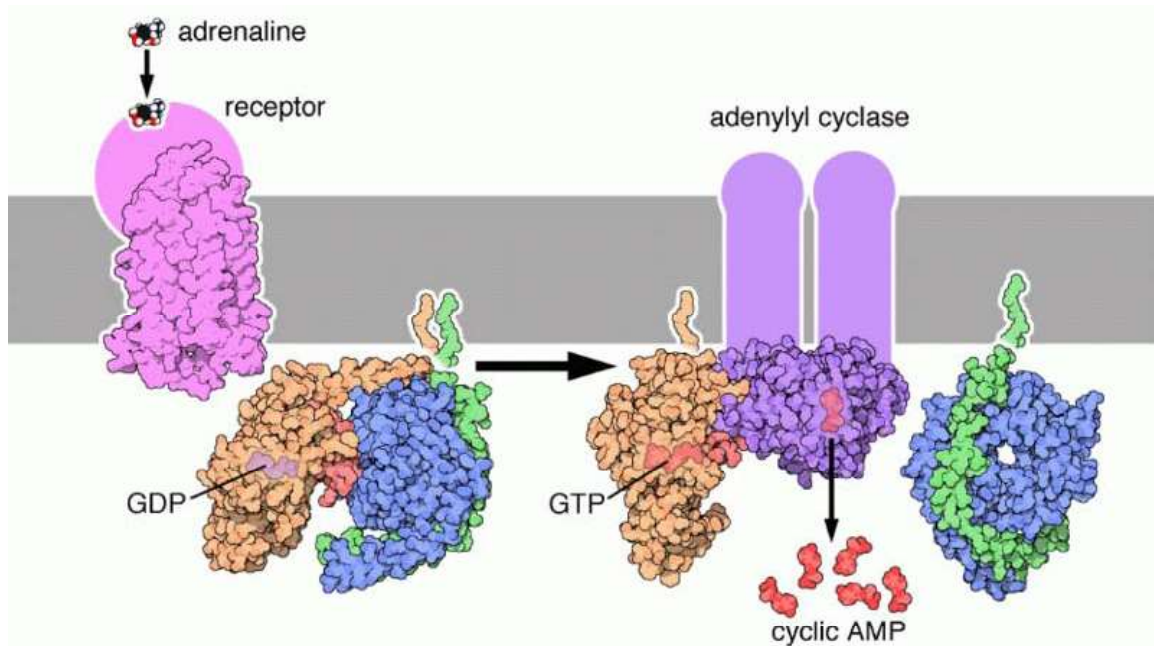
## **Nitric oxide**

Nitric oxide (NO) can act as a second messenger. Nitric oxide gas is a free radical that freely diffuses through the plasma membrane and affects nearby cells. NO is made from arginine and oxygen by the enzyme NO synthase, with citrulline as a by-product. NO works mainly through activation of its target receptor, the enzyme soluble guanylate

cyclase, which, when activated, produces the second messenger cyclic-guanosine monophosphate (cGMP). NO can also act through covalent modification of proteins or their metal co-factors. Some of these modifications are reversible and work through a redox mechanism. NO is toxic in high concentrations, and is thought to cause damage during stroke.

NO is involved in a number of functions, including relaxation of blood vessels; regulation of exocytosis of neurotransmitters; cellular immune response; modulation of the Hair Cycle; production and maintenance of penile erections; and activation of apoptosis by initiating signals that lead to H2AX phosphorylation.

### **Major pathway examples**

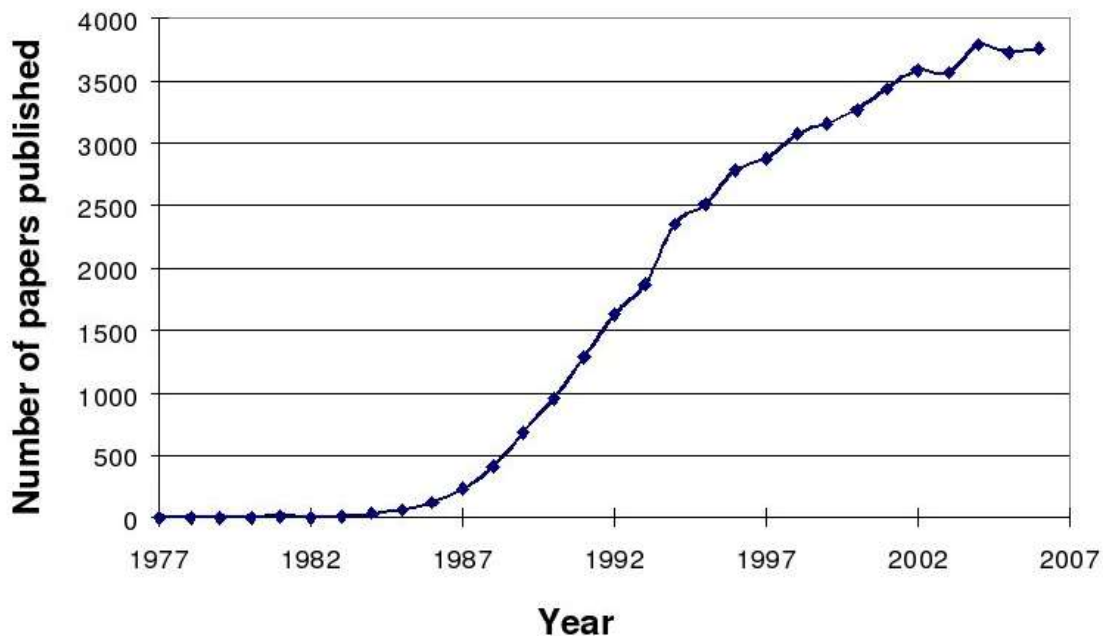


**Mechanism of cAMP dependent signaling.** In this figure, the neurotransmitter epinephrine (adrenaline) and its receptor (pink) is used as an example. The activated receptor releases the G<sub>s</sub> alpha protein (tan) from the beta and gamma subunits (blue and green) in the heterotrimeric G-protein complex. The activated G<sub>s</sub> alpha protein in turn activates adenylyl cyclase (purple) that converts ATP into the second messenger cAMP.

- cAMP dependent pathway: In humans, cAMP works by activating protein kinase A (PKA, cAMP-dependent protein kinase), and thus, further effects mainly depend on cAMP-dependent protein kinase, which vary based on the type of cell.
- MAPK/ERK pathway: A pathway that couples intracellular responses to the binding of growth factors to cell surface receptors. This pathway is very complex and includes many protein components. The basic pathway shown in the figure (to the right) and described below includes the major components of the pathway. In many cell types, activation of this pathway promotes cell division.

- IP<sub>3</sub>/DAG pathway: PLC cleaves the phospholipid phosphatidylinositol 4,5-bisphosphate (PIP<sub>2</sub>) yielding diacyl glycerol (DAG) and inositol 1,4,5-triphosphate (IP<sub>3</sub>). DAG remains bound to the membrane, and IP<sub>3</sub> is released as a soluble structure into the cytosol. IP<sub>3</sub> then diffuses through the cytosol to bind to IP<sub>3</sub> receptors, particular calcium channels in the endoplasmic reticulum (ER). These channels are specific to calcium and only allow the passage of calcium to move through. This causes the cytosolic concentration of Calcium to increase, causing a cascade of intracellular changes and activity. In addition, calcium and DAG together works to activate PKC, which goes on to phosphorylate other molecules, leading to altered cellular activity. End effects include taste, manic depression, tumor promotion, etc.

## History



**Occurrence of the term “signal transduction”** The total number of papers published in each year since 1977 containing the phrase *signal transduction* in either their title or abstract section are plotted. These figures were extracted through an analysis of the papers contained within the MEDLINE database.

The earliest scientific paper recorded in the MEDLINE database as containing the specific term *signal transduction* within its text was published in 1972.

Some articles published before 1977 use the term *signal transmission* or *sensory transduction* in their titles or abstracts but it was not until 1977 that papers began to be published with the specific term *signal transduction* in their abstracts, and it was not until 1979 that the term appeared within a paper title.. One source attributes the widespread use of the term *signal transduction* to a 1980 review article by Rodbell. As can be seen

from the graph to the right, research papers directly addressing signal transduction processes began to appear in large numbers in the scientific literature in the late 1980s and early 1990s.

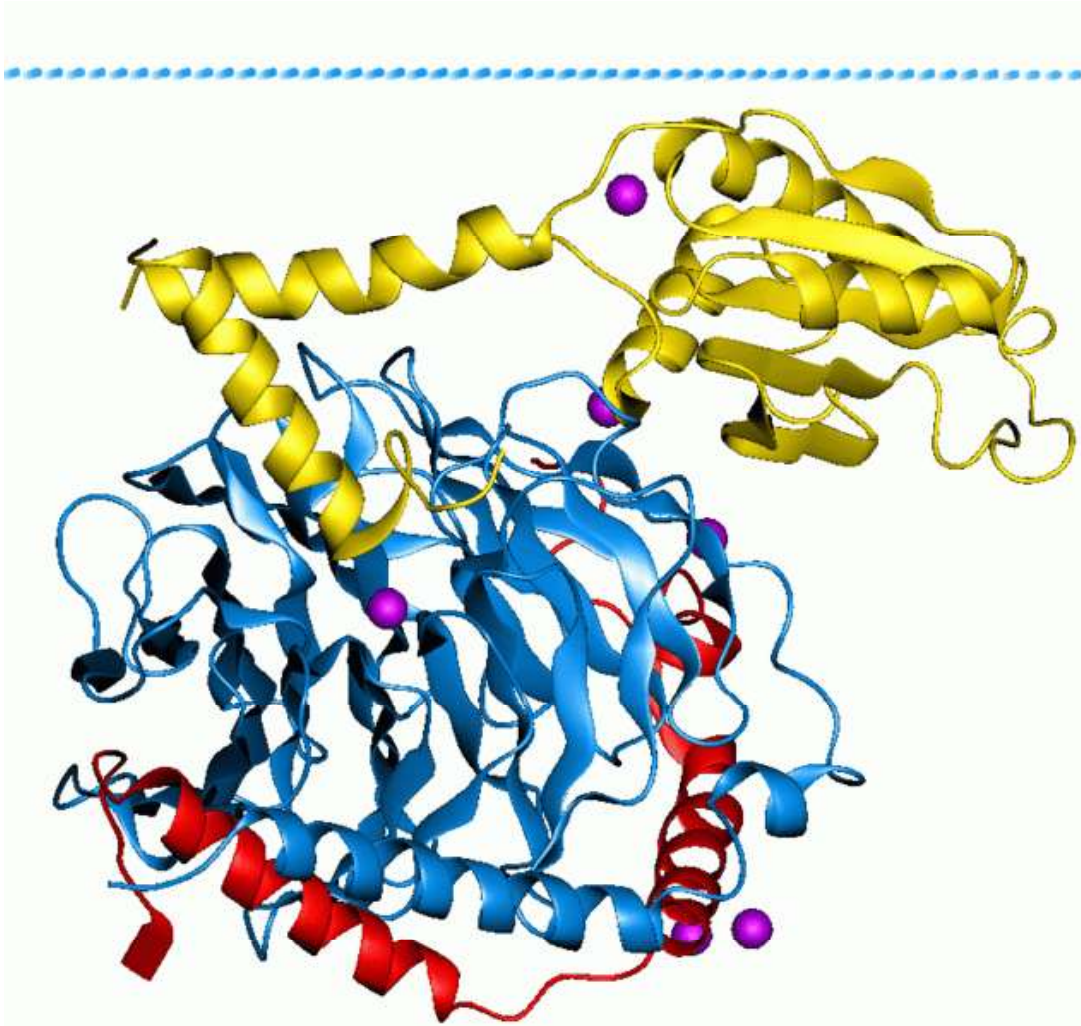
One notable early discovery in the field of signal transduction was the link Rodbell made between metabolic regulation and the activity of GTP and GTP-binding proteins. The current understanding of signal transduction processes reflects contributions made over many years by research groups all over the world.

A total of 48,377 scientific papers related to signal transduction were published in 1977; of these, 11,211 were reviews of other papers.

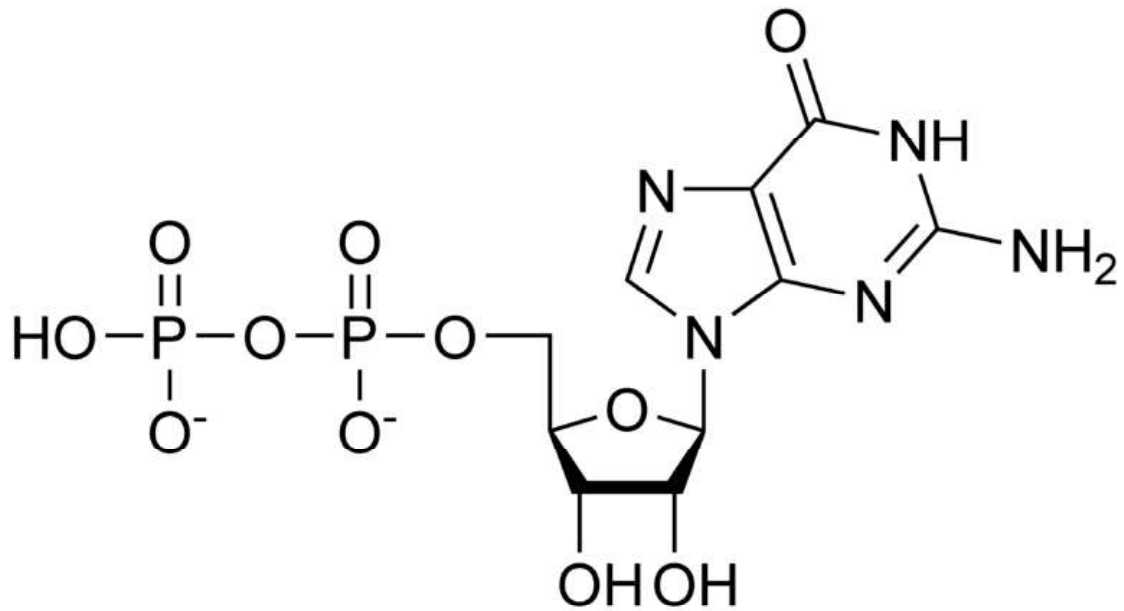
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## Chapter 7

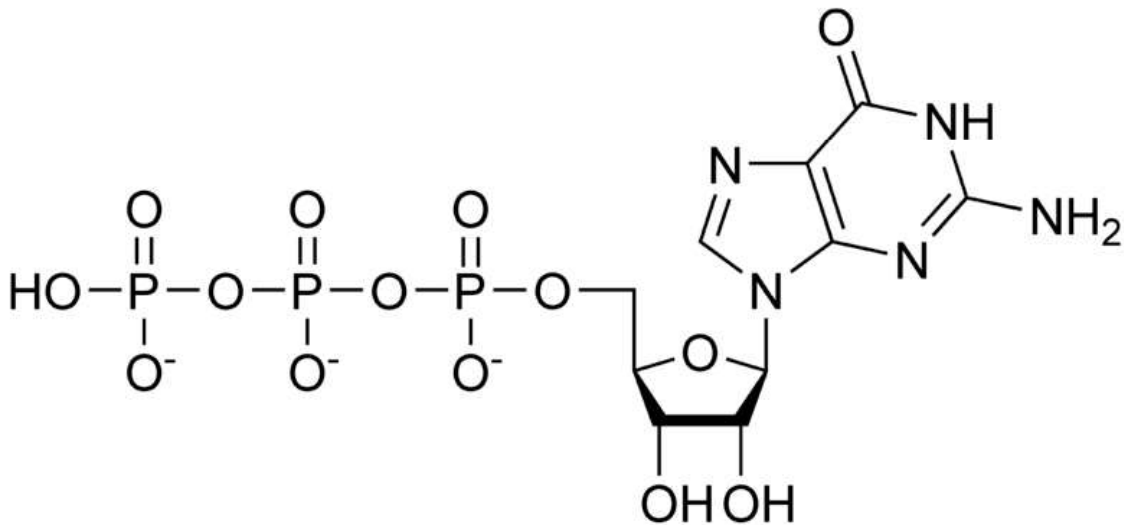
# G protein



Phosducin- transducin beta-gamma complex. Beta and gamma subunits of G-protein are shown by blue and red, respectively.



Guanosine diphosphate



Guanosine triphosphate

**G proteins (guanine nucleotide-binding proteins)** are a family of proteins involved in transmitting chemical signals outside the cell, and causing changes inside the cell. They communicate signals from many hormones, neurotransmitters, and other signaling factors.

G protein-coupled receptors are transmembrane receptors. Signal molecules bind to a domain located outside the cell. An intracellular domain activates a G protein. The G

protein activates a cascade of further compounds, and finally causes a change downstream in the cell.

G protein complexes bind to phosphate groups. They function as molecular switches. When they are attached to a complex with three phosphate groups (guanosine triphosphate [GTP]), they turn on. When they are attached to a complex with only two phosphate groups (guanosine diphosphate [GDP]), they turn off.

G proteins regulate metabolic enzymes, ion channels, transporters, and other parts of the cell machinery, controlling transcription, motility, contractility, and secretion, which in turn regulate systemic functions such as embryonic development, learning and memory, and homeostasis.

G proteins were discovered when Alfred G. Gilman and Martin Rodbell investigated stimulation of cells by adrenaline. They found that, when adrenaline binds to a receptor, the receptor does not stimulate enzymes directly. Instead, the receptor stimulates a G protein, which stimulates an enzyme. An example is adenylate cyclase, which produces the second messenger cyclic AMP. For this discovery, they won the 1994 Nobel Prize in Physiology or Medicine.

G proteins belong to the larger group of enzymes called GTPases.

### **Function**

G proteins are important signal transducing molecules in cells. "Malfunction of GPCR [G Protein-Coupled Receptor] signaling pathways are involved in many diseases, such as diabetes, blindness, allergies, depression, cardiovascular defects, and certain forms of cancer. It is estimated that more than half of the modern drugs' cellular targets are GPCRs."

The human genome encodes roughly 950 G protein-coupled receptors, which detect photons (light), hormones, growth factors, drugs, and other endogenous ligands. Approximately 150 of the GPCRs found in the human genome have unknown functions.

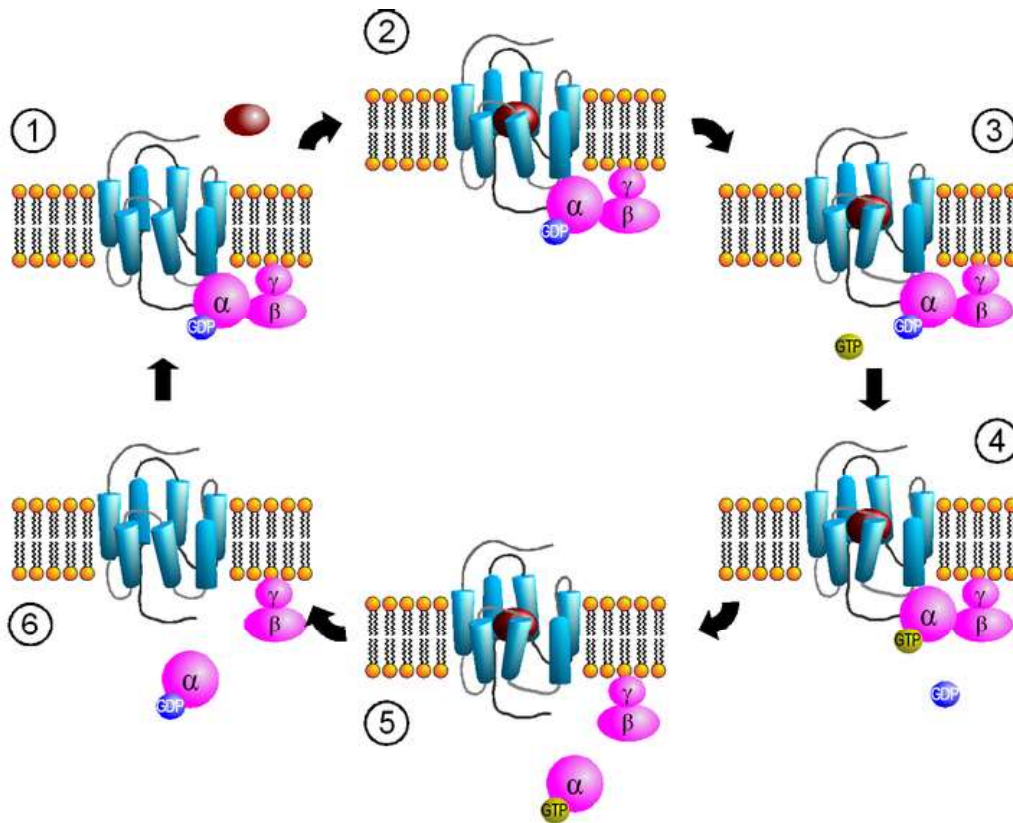
### **Types of G protein signaling**

G protein can refer to two distinct families of proteins. Heterotrimeric G proteins, sometimes referred to as the "large" G proteins that are activated by G protein-coupled receptors and made up of alpha ( $\alpha$ ), beta ( $\beta$ ), and gamma ( $\gamma$ ) subunits. There are also "*small*" G proteins (20-25kDa) that belong to the Ras superfamily of small GTPases. These proteins are homologous to the alpha ( $\alpha$ ) subunit found in heterotrimers, and are in fact monomeric. However, they also bind GTP and GDP and are involved in signal transduction.

## Heterotrimeric G proteins

Different types of heterotrimeric G proteins share a common mechanism. They are activated in response to a conformational change in the G protein-coupled receptor, exchange GDP for GTP, and dissociate to activate other proteins in the signal transduction pathway. The specific mechanisms, however, differ among the types.

### Common mechanism



Activation cycle of G-proteins by G-protein-coupled receptors

Receptor-activated G proteins are bound to the inside surface of the cell membrane. They consist of the  $G_\alpha$  and the tightly associated  $G_{\beta\gamma}$  subunits. There are many classes of  $G_\alpha$  subunits:  $G_s\alpha$  (G stimulatory),  $G_i\alpha$  (G inhibitory),  $G_o\alpha$  (G other),  $G_{q/11\alpha}$ , and  $G_{12/13\alpha}$  are some examples. They behave differently in the recognition of the effector, but share a similar mechanism of activation.

### Activation

When a ligand activates the G protein-coupled receptor, it induces a conformational change in the receptor that allows the receptor to function as a guanine nucleotide exchange factor (GEF) that exchanges GDP for GTP on the  $G_\alpha$  subunit. In the traditional view of heterotrimeric protein activation, this exchange triggers the dissociation of the  $G_\alpha$

subunit, bound to GTP, from the  $G_{\beta\gamma}$  dimer and the receptor. However, models that suggest molecular rearrangement, reorganization, and pre-complexing of effector molecules are beginning to be accepted. Both  $G_{\alpha}$ -GTP and  $G_{\beta\gamma}$  can then activate different *signaling cascades* (or *second messenger pathways*) and effector proteins, while the receptor is able to activate the next G protein.

### **Termination**

The  $G_{\alpha}$  subunit will eventually hydrolyze the attached GTP to GDP by its inherent enzymatic activity, allowing it to re-associate with  $G_{\beta\gamma}$  and starting a new cycle. A group of proteins called Regulator of G protein signalling (RGSs), act as GTPase-activating proteins (GAPs), specific for  $G_{\alpha}$  subunits. These proteins act to accelerate hydrolysis of GTP to GDP and terminate the transduced signal. In some cases, the effector itself may possess intrinsic GAP activity, which helps deactivate the pathway. This is true in the case of phospholipase C beta, which possesses GAP activity within its C-terminal region. This is an alternate form of regulation for the  $G_{\alpha}$  subunit.

### **Specific mechanisms**

- $G_{\alpha s}$  activates the cAMP-dependent pathway by stimulating the production of cAMP from ATP. This is accomplished by direct stimulation of the membrane-associated enzyme adenylyl cyclase. cAMP acts as a second messenger that goes on to interact with and activate protein kinase A (PKA). PKA can then phosphorylate myriad downstream targets.
- $G_{\alpha i}$  inhibits the production of cAMP from ATP.
- $G_{\alpha q/11}$  stimulates membrane-bound phospholipase C beta, which then cleaves  $PIP_2$  (a minor membrane phosphoinositol) into two second messengers, IP3 and diacylglycerol (DAG).
- $G_{\alpha 12/13}$  are involved in Rho family GTPase signaling (through RhoGEF superfamily) and control cell cytoskeleton remodeling, thus regulating cell migration.
- $G_{\beta\gamma}$  sometimes also have active functions, e.g., coupling to and activating GIRK Channels.

### **Small GTPases**

Small GTPases also bind GTP and GDP and are involved in signal transduction. These proteins are homologous to the alpha ( $\alpha$ ) subunit found in heterotrimers, but exist as monomers. They are small (20-kDa to 25-kDa) proteins that bind to guanosine triphosphate (GTP). This family of proteins is homologous to Ras GTPases and is also called the Ras superfamily GTPases.

## ***Lipidation***

In order to associate with the inner leaflet of the plasma membrane, many G proteins and small GTPases are lipidated, that is, covalently modified with lipid extensions. They may be myristoylated, palmitoylated or prenylated.

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## Chapter 8

# Staining



A stained histologic specimen, sandwiched between a glass microscope slide and coverslip, mounted on the stage of a light microscope.

**Staining** is an auxiliary technique used in microscopy to enhance contrast in the microscopic image. Stains and dyes are frequently used in biology and medicine to highlight structures in biological tissues for viewing, often with the aid of different microscopes. Stains may be used to define and examine bulk tissues (highlighting, for example, muscle fibers or connective tissue), cell populations (classifying different blood cells, for instance), or organelles within individual cells.

In biochemistry it involves adding a class-specific (DNA, proteins, lipids, carbohydrates) dye to a substrate to qualify or quantify the presence of a specific compound. Staining and fluorescent tagging can serve similar purposes. Biological staining is also used to mark cells in flow cytometry, and to flag proteins or nucleic acids in gel electrophoresis.

Staining is not limited to biological materials, it can also be used to study the morphology of other materials for example the lamellar structures of semi-crystalline polymers or the domain structures of block copolymers.

## **In vivo vs In vitro**

*In vivo* staining is the process of dyeing living tissues—*in vivo* means "in life" (compare with *in vitro* staining). By causing certain cells or structures to take on contrasting colour(s), their form (morphology) or position within a cell or tissue can be readily seen and studied. The usual purpose is to reveal cytological details that might otherwise not be apparent; however, staining can also reveal where certain chemicals or specific chemical reactions are taking place within cells or tissues.

*In vitro* staining involves colouring cells or structures that are no longer living. Certain stains are often combined to reveal more details and features than a single stain alone. Combined with specific protocols for fixation and sample preparation, scientists and physicians can use these standard techniques as consistent, repeatable diagnostic tools. A counterstain is stain that makes cells or structures more visible, when not completely visible with the principal stain.

- For example, crystal violet stains only Gram-positive bacteria in Gram staining. A safranin counterstain is applied which stains all cells, allowing the identification of Gram-negative bacteria as well.

Often these stains are called vital stains. They are introduced to the organism while the cells are still living. However, these stains are eventually toxic to the organism, some more so than others. To achieve desired effects, the stains are used in very dilute solutions ranging from 1:5000 to 1:500000 (Howey, 2000). Note that many stains may be used in both living and fixed cells.

## **In vitro methods**

### **Preparation**

The preparatory steps involved depend on the type of analysis planned; some or all of the following procedures may be required.

**Fixation**—which may itself consist of several steps—aims to preserve the shape of the cells or tissue involved as much as possible. Sometimes heat fixation is used to kill, adhere, and alter the specimen so it will accept stains. Most chemical fixatives (chemicals causing fixation) generate chemical bonds between proteins and other substances within

the sample, increasing their rigidity. Common fixatives include formaldehyde, ethanol, methanol, and/or picric acid. Pieces of tissue may be embedded in paraffin wax to increase their mechanical strength and stability and to make them easier to cut into thin slices.

**Permeabilization** involves treatment of cells with (usually) a mild surfactant. This treatment will dissolve the cell membranes, and allow larger dye molecules access to the cell's interior.

**Mounting** usually involves attaching the samples to a glass microscope slide for observation and analysis. In some cases, cells may be grown directly on a slide. For samples of loose cells (as with a blood smear or a pap smear) the sample can be directly applied to a slide. For larger pieces of tissue, thin sections (slices) are made using a microtome; these slices can then be mounted and inspected.

### **Staining proper**

At its simplest, the actual staining process may involve immersing the sample (before or after fixation and mounting) in dye solution, followed by rinsing and observation. Many dyes, however, require the use of a mordant: a chemical compound which reacts with the stain to form an insoluble, coloured precipitate. When excess dye solution is washed away, the mordanted stain remains.

Most of the dyes commonly used in microscopy are available as **certified stains**. This means that samples of the manufacturer's batch have been tested by an independent body, the Biological Stain Commission and found to meet or exceed certain standards of purity, dye content and performance in staining techniques. These standards are published in detail in the journal *Biotechnic & Histochemistry*. Many dyes are inconsistent in composition from one supplier to another. The use of certified stains eliminates a source of unexpected results.

### **Negative staining**

A simple staining method for bacteria which is usually successful even when the "positive staining" methods detailed below fail, is to employ a negative stain. This can be achieved simply by smearing the sample on to the slide, followed by an application of nigrosin (a black synthetic dye) or Indian ink (an aqueous suspension of carbon particles). After drying, the microorganisms may be viewed in bright field microscopy as lighter inclusions well-contrasted against the dark environment surrounding them. Note: negative staining is a mild technique which may not destroy the microorganisms therefore it is unsuitable for studying pathogens.

## Specific techniques

### Gram staining

Gram staining is used to determine gram status to classify bacteria broadly. It is based on the composition of their cell wall. Gram staining uses crystal violet to stain cell walls, iodine as a mordant, and a fuchsin or safranin counterstain to mark all bacteria. Gram status is important in medicine; the presence or absence of a cell wall will change the bacterium's susceptibility to some antibiotics.

Gram-positive bacteria stain dark blue or violet. Their cell wall is typically rich with peptidoglycan and lacks the secondary membrane and lipopolysaccharide layer found in Gram-negative bacteria.

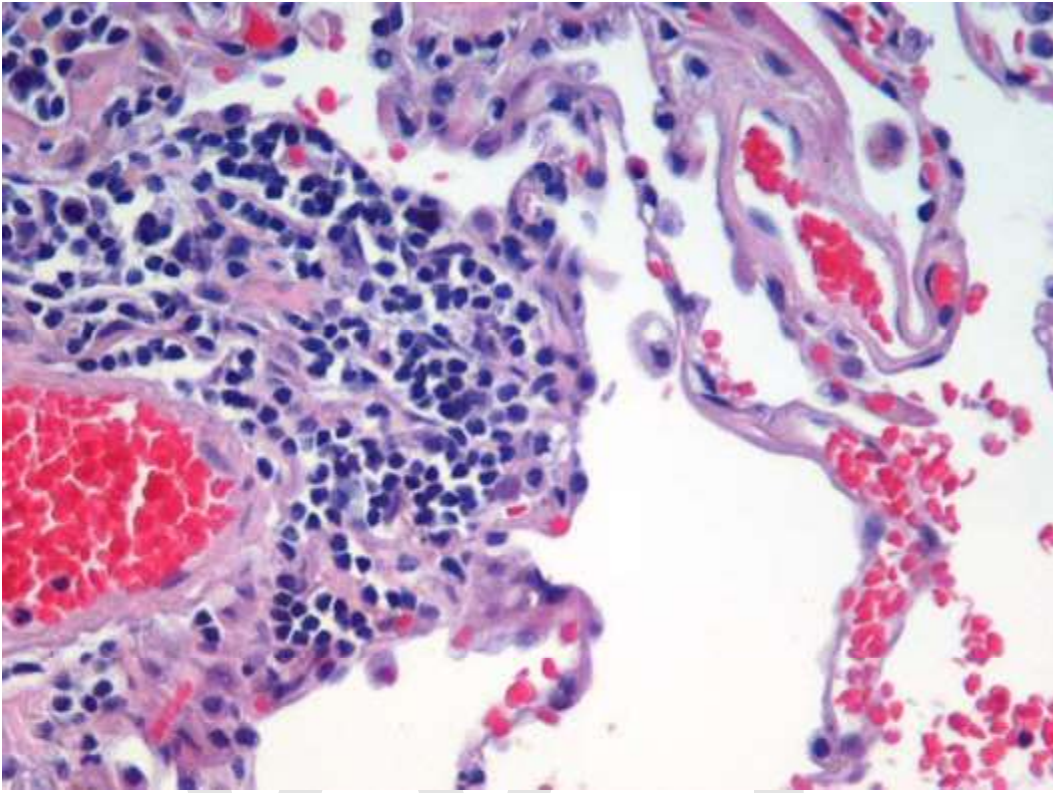
On most Gram-stained preparations, Gram-negative organisms will appear red or pink because they are counterstained. Due to presence of higher lipid content, after alcohol-treatment, the porosity of the cell wall increases, hence the CVI complex (Crystal violet - Iodine) can pass through. Thus, the primary stain is not retained. Also, in contrast to most Gram-positive bacteria, Gram-negative bacteria have only a few layers of peptidoglycan and a secondary cell membrane made primarily of lipopolysaccharide.

### Ziehl-Neelsen stain

Ziehl-Neelsen staining is used to stain species of *Mycobacterium tuberculosis* that do not stain with the standard laboratory staining procedures like Gram staining.

The stains used are the red coloured Carbol fuchsin that stains the bacteria and a counter stain like Methylene blue or Malachite green.

## Haematoxylin and eosin (H&E) staining



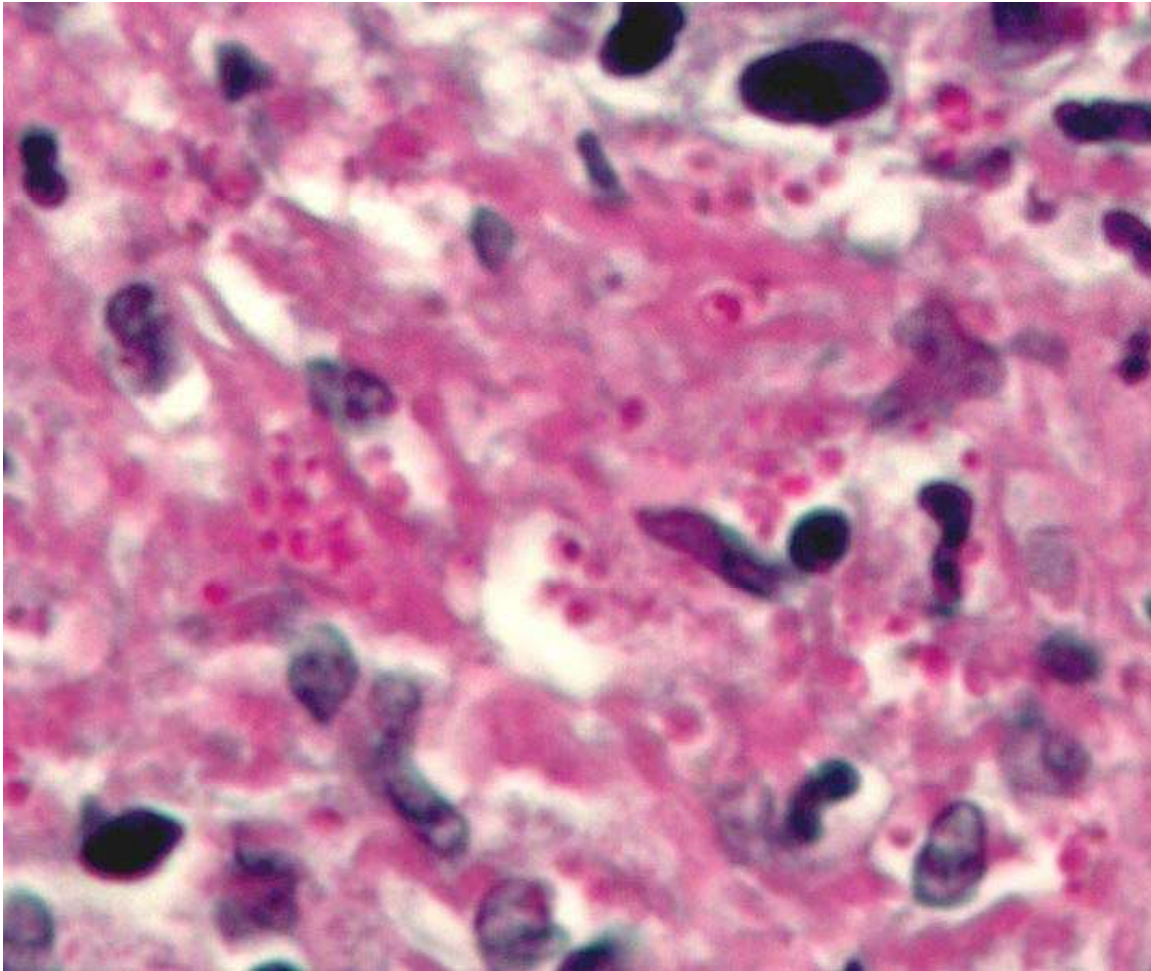
Microscopic view of a histologic specimen of human lung tissue stained with hematoxylin and eosin.

Haematoxylin and eosin staining protocol is used frequently in histology to examine thin sections of tissue. Haematoxylin stains cell nuclei blue, while eosin stains cytoplasm, connective tissue and other extracellular substances pink or red. Eosin is strongly absorbed by red blood cells, colouring them bright red. In a skilfully made H & E preparation the red blood cells are almost orange, and collagen and cytoplasm (especially muscle) acquire different shades of pink. When the staining is done by a machine, the subtle differences in eosinophilia are often lost.

### **Papanicolaou staining**

Papanicolaou staining, or Pap staining, is a frequently used method for examining cell samples from various bodily secretions. It is frequently used to stain Pap smear specimens. It uses a combination of haematoxylin, Orange G, eosin Y, Light Green SF yellowish, and sometimes Bismarck Brown Y.

## PAS staining



PAS diastase showing the fungus Histoplasma.

Periodic acid-Schiff staining is used to mark carbohydrates (glycogen, glycoprotein, proteoglycans). It is used to distinguish different types of glycogen storage diseases.

## Masson's trichrome

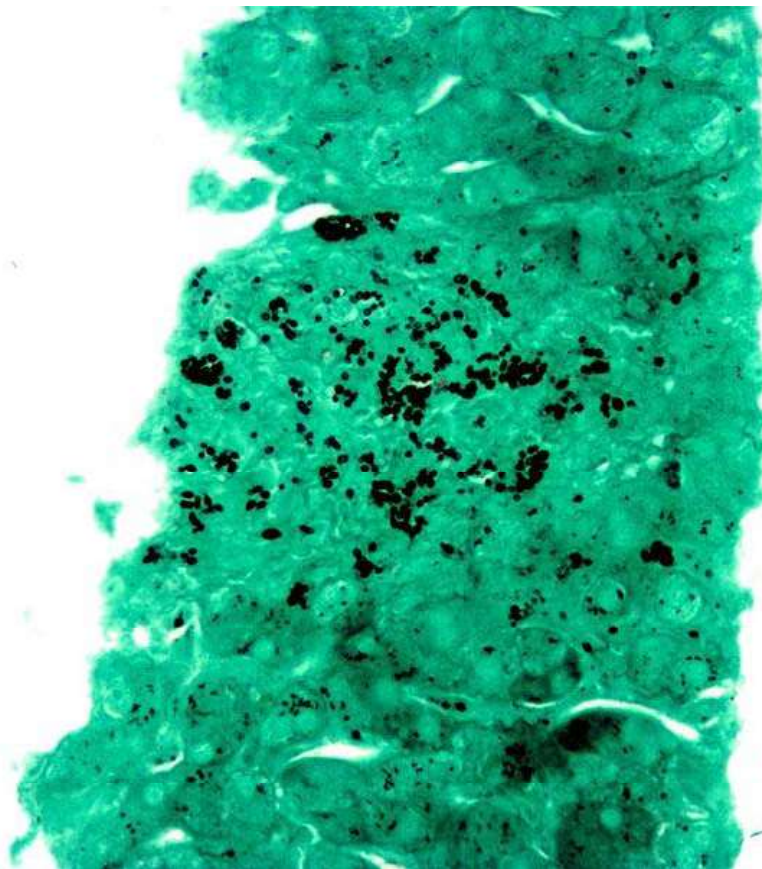
Masson's trichrome is (as the name implies) a three-colour staining protocol. The recipe has evolved from Masson's original technique for different specific applications, but all are well-suited to distinguish cells from surrounding connective tissue. Most recipes will produce red keratin and muscle fibers, blue or green staining of collagen and bone, light red or pink staining of cytoplasm, and black cell nuclei.

## Romanowsky stains

The Romanowsky stains are all based on a combination of eosinate (chemically reduced eosin) and methylene blue (sometimes with its oxidation products azure A and azure B). Common variants include Wright's stain, Jenner's stain, Leishman stain and Giemsa stain.

All are used to examine blood or bone marrow samples. They are preferred over H&E for inspection of blood cells because different types of leukocytes (white blood cells) can be readily distinguished. All are also suited to examination of blood to detect blood-borne parasites like malaria.

## Silver staining



Gömöri methenamine silver stain demonstrating histoplasma (black round balls).

Silver staining is the use of silver to stain histologic sections. This kind of staining is important especially to show proteins (for example type III collagen) and DNA. It is used to show both substances inside and outside cells. Silver staining is also used in temperature gradient gel electrophoresis.

Some cells are *argentaffin*. These reduce silver solution to metallic silver after formalin fixation. This method was discovered by Italian Camillo Golgi, by using a reaction

between silver nitrate and potassium dichromate, thus precipitating silver chromate in some cells. Other cells are *argyrophilic*. These reduce silver solution to metallic silver after being exposed to the stain that contains a reductant, for example hydroquinone or formalin.

### **Sudan staining**

Sudan staining is the use of Sudan dyes to stain sudanophilic substances, usually lipids. Sudan III, Sudan IV, Oil Red O, and Sudan Black B are often used. Sudan staining is often used to determine the level of fecal fat to diagnose steatorrhea.

### **Conklin's staining**

Special technique designed for staining true endospores with the use of malachite green dye, once stained, they do not decolourize.

## ***Common biological stains***

Different stains react or concentrate in different parts of a cell or tissue, and these properties are used to advantage to reveal specific parts or areas. Some of the most common biological stains are listed below. Unless otherwise marked, all of these dyes may be used with fixed cells and tissues; vital dyes (suitable for use with living organisms) are noted.

### **Acridine orange**

Acridine orange (AO) is a nucleic acid selective fluorescent cationic dye useful for cell cycle determination. It is cell-permeable, and interacts with DNA and RNA by intercalation or electrostatic attractions. When bound to DNA, it is very similar spectrally to fluorescein. Like fluorescein, it is also useful as a non-specific stain for backlighting conventionally stained cells on the surface of a solid sample of tissue (fluorescence backlighting staining).

### **Bismarck brown**

Bismarck brown (also Bismarck brown Y or Manchester brown) imparts a yellow colour to acid mucins. Bismarck brown may be used with live cells.

### **Carmin**

Carmin is an intensely red dye which may be used to stain glycogen, while Carmin alum is a nuclear stain. Carmin stains require the use of a mordant, usually aluminum.

## **Coomassie blue**

Coomassie blue (also brilliant blue) nonspecifically stains proteins a strong blue colour. It is often used in gel electrophoresis.

## **Crystal violet**

Crystal violet, when combined with a suitable mordant, stains cell walls purple. Crystal violet is an important component in Gram staining.

## **DAPI**

DAPI is a fluorescent nuclear stain, excited by ultraviolet light and showing strong blue fluorescence when bound to DNA. DAPI binds with A=T rich repeats of chromosomes. DAPI is also not visible with regular transmission microscopy. It may be used in living or fixed cells. DAPI-stained cells are especially appropriate for cell counting.

## **Eosin**

Eosin is most often used as a counterstain to haematoxylin, imparting a pink or red colour to cytoplasmic material, cell membranes, and some extracellular structures. It also imparts a strong red colour to red blood cells. Eosin may also be used as a counterstain in some variants of Gram staining, and in many other protocols. There are actually two very closely related compounds commonly referred to as eosin. Most often used is eosin Y (also known as eosin Y ws or eosin yellowish); it has a very slightly yellowish cast. The other eosin compound is eosin B (eosin bluish or imperial red); it has a very faint bluish cast. The two dyes are interchangeable, and the use of one or the other is more a matter of preference and tradition.

## **Ethidium bromide**

Ethidium bromide intercalates and stains DNA, providing a fluorescent red-orange stain. Although it will not stain healthy cells, it can be used to identify cells that are in the final stages of apoptosis - such cells have much more permeable membranes. Consequently, ethidium bromide is often used as a marker for apoptosis in cells populations and to locate bands of DNA in gel electrophoresis. The stain may also be used in conjunction with acridine orange (AO) in viable cell counting. This EB/AO combined stain causes live cells to fluoresce green whilst apoptotic cells retain the distinctive red-orange fluorescence.

## **Acid fuchsin**

Acid fuchsin may be used to stain collagen, smooth muscle, or mitochondria. Acid fuchsin is used as the nuclear and cytoplasmic stain in Mallory's trichrome method. Acid fuchsin stains cytoplasm in some variants of Masson's trichrome. In Van Gieson's picro-

fuch sine, acid fuch sine imparts its red colour to collagen fibres. Acid fuch sine is also a traditional stain for mitochondria (Altmann's method).

### **Haematoxylin**

Haematoxylin (hematoxylin in North America) is a nuclear stain. Used with a mordant, haematoxylin stains nuclei blue-violet or brown. It is most often used with eosin in H&E (haematoxylin and eosin) staining—one of the most common procedures in histology.

### **Hoechst stains**

Hoechst is a *bis*-benzimidazole derivative compound which binds to the *minor groove* of DNA. Often used in fluorescence microscopy for DNA staining, Hoechst stains appear yellow when dissolved in aqueous solutions and emit blue light under UV excitation. There are two major types of Hoechst: *Hoechst 33258* and *Hoechst 33342*. The two compounds are functionally similar, but with a little difference in structure. Hoechst 33258 contains a terminal hydroxyl group and is thus more soluble in aqueous solution, however this characteristic reduces its ability to penetrate the plasma membrane. Hoechst 33342 contains a ethyl substitution on the terminal hydroxyl group (i.e. an ethylether group) making it more hydrophobic for easier plasma membrane passage

### **Iodine**

Iodine is used in chemistry as an indicator for starch. When starch is mixed with iodine in solution, an intensely dark blue colour develops, representing a starch/iodine complex. Starch is a substance common to most plant cells and so a weak iodine solution will stain starch present in the cells. Iodine is one component in the staining technique known as Gram staining, used in microbiology. Lugol's solution or Lugol's iodine (IKI) is a brown solution that turns black in the presence of starches and can be used as a cell stain, making the cell nuclei more visible. Iodine is also used as a mordant in Gram's staining, it enhances dye to enter through the pore present in the cell wall/membrane.

### **Malachite green**

Malachite green (also known as diamond green B or victoria green B) can be used as a blue-green counterstain to safranin in the Gimenez staining technique for bacteria. It also can be used to directly stain spores.

### **Methyl green**

Methyl green is used commonly with bright-field microscopes to dye the chromatin of cells so that they are more easily viewed.

## **Methylene blue**

Methylene blue is used to stain animal cells, such as human cheek cells, to make their nuclei more observable. Also used to staining the blood film and used in cytology.

## **Neutral red**

Neutral red (or toluylene red) stains Nissl substance red. It is usually used as a counterstain in combination with other dyes.

## **Nile blue**

Nile blue (or Nile blue A) stains nuclei blue. It may be used with living cells.

## **Nile red**

Nile red (also known as Nile blue oxazone) is formed by boiling Nile blue with sulfuric acid. This produces a mix of Nile red and Nile blue. Nile red is a lipophilic stain; it will accumulate in lipid globules inside cells, staining them red. Nile red can be used with living cells. It fluoresces strongly when partitioned into lipids, but practically not at all in aqueous solution.

## **Osmium tetroxide (formal name: osmium tetroxide)**

Osmium tetroxide is used in optical microscopy to stain lipids. It dissolves in fats, and is reduced by organic materials to elemental osmium, an easily visible black substance.

## **Rhodamine**

Rhodamine is a protein specific fluorescent stain commonly used in fluorescence microscopy.

## **Safranin**

Safranin (or Safranin O) is a nuclear stain. It produces red nuclei, and is used primarily as a counterstain. Safranin may also be used to give a yellow colour to collagen.

## ***Stainability of tissues***

Positive affinity for a specific stain may be designated by the suffix *-philic*. For example, tissues that stain with an azure dye may be referred to as azurophilic. This may also be used for more generalized staining properties, such as acidophilic for tissues that stain by acidic stains (most notably eosin), basophilic when staining in basic dyes and *amphophilic* when staining with either acid or basic dyes. In contrast, Chromophobic tissues do not take up coloured dye readily.

## ***Electron microscopy***

As in light microscopy, stains can be used to enhance contrast in transmission electron microscopy. Electron-dense compounds of heavy metals are typically used.

### **Phosphotungstic acid**

Phosphotungstic acid is a common negative stain for viruses, nerves, polysaccharides, and other biological tissue materials.

### **Osmium tetroxide**

Osmium tetroxide is used in optical microscopy to stain lipids. It dissolves in fats, and is reduced by organic materials to elemental osmium, an easily visible black substance. Because it is a heavy metal that absorbs electrons, it is perhaps the most common stain used for morphology in biological electron microscopy. It is also used for the staining of various polymers for the study of their morphology by TEM.  $\text{OsO}_4$  is very volatile and extremely toxic. It is a strong oxidizing agent as the osmium has an oxidation number of +8. It aggressively oxidizes many materials, leaving behind a deposit of non-volatile osmium in a lower oxidation state.

### **Ruthenium tetroxide**

Ruthenium tetroxide is equally volatile and even more aggressive than osmium tetroxide and able to stain even materials that resist the osmium stain, e.g. polyethylene.

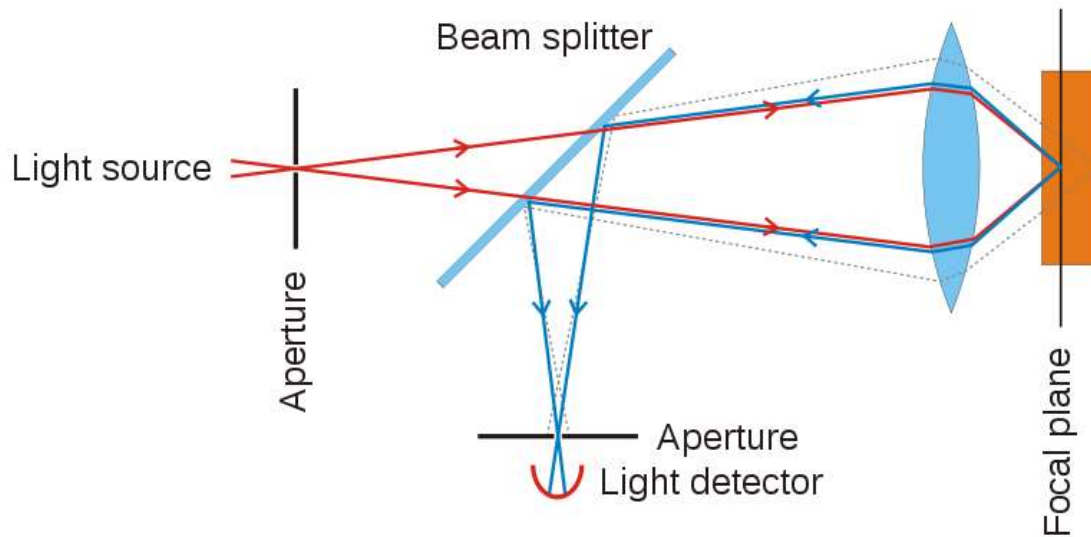
Other chemicals used in electron microscopy staining include: ammonium molybdate, cadmium iodide, carbonyl dihydrazide, ferric chloride, hexamine, indium trichloride, lanthanum nitrate, lead acetate, lead citrate, lead(II) nitrate, periodic acid, phosphomolybdic acid, potassium ferricyanide, potassium ferrocyanide, ruthenium red, silver nitrate, silver proteinate, sodium chloroaurate, thallium nitrate, thiosemicarbazide, uranyl acetate, uranyl nitrate, and vanadyl sulfate.

## Chapter 9

# Confocal Laser Scanning Microscopy

**Confocal laser scanning microscopy (CLSM or LSCM)** is a technique for obtaining high-resolution optical images with depth selectivity. The key feature of confocal microscopy is its ability to acquire in-focus images from selected depths, a process known as optical sectioning. Images are acquired point-by-point and reconstructed with a computer, allowing three-dimensional reconstructions of topologically complex objects. For opaque specimens, this is useful for surface profiling, while for non-opaque specimens, interior structures can be imaged. For interior imaging, the quality of the image is greatly enhanced over simple microscopy because image information from multiple depths in the specimen is not superimposed. A conventional microscope "sees" as far into the specimen as the light can penetrate, while a confocal microscope only images one depth level at a time. In effect, the CLSM achieves a controlled and highly limited depth of focus. The principle of confocal microscopy was originally patented by Marvin Minsky in 1957, but it took another thirty years and the development of lasers for CLSM to become a standard technique toward the end of the 1980s. In 1978, Thomas and Christoph Cremer designed a laser scanning process, which scans the three dimensional surface of an object point-by-point by means of a focused laser beam, and creates the over-all picture by electronic means similar to those used in scanning electron microscopes. This CLSM design combined the laser scanning method with the 3D detection of biological objects labeled with fluorescent markers for the first time. During the next decade, confocal fluorescence microscopy was developed into a fully mature technology, in particular by groups working at the University of Amsterdam and the European Molecular Biology Laboratory (EMBL) in Heidelberg and their industry partners.

## Image formation



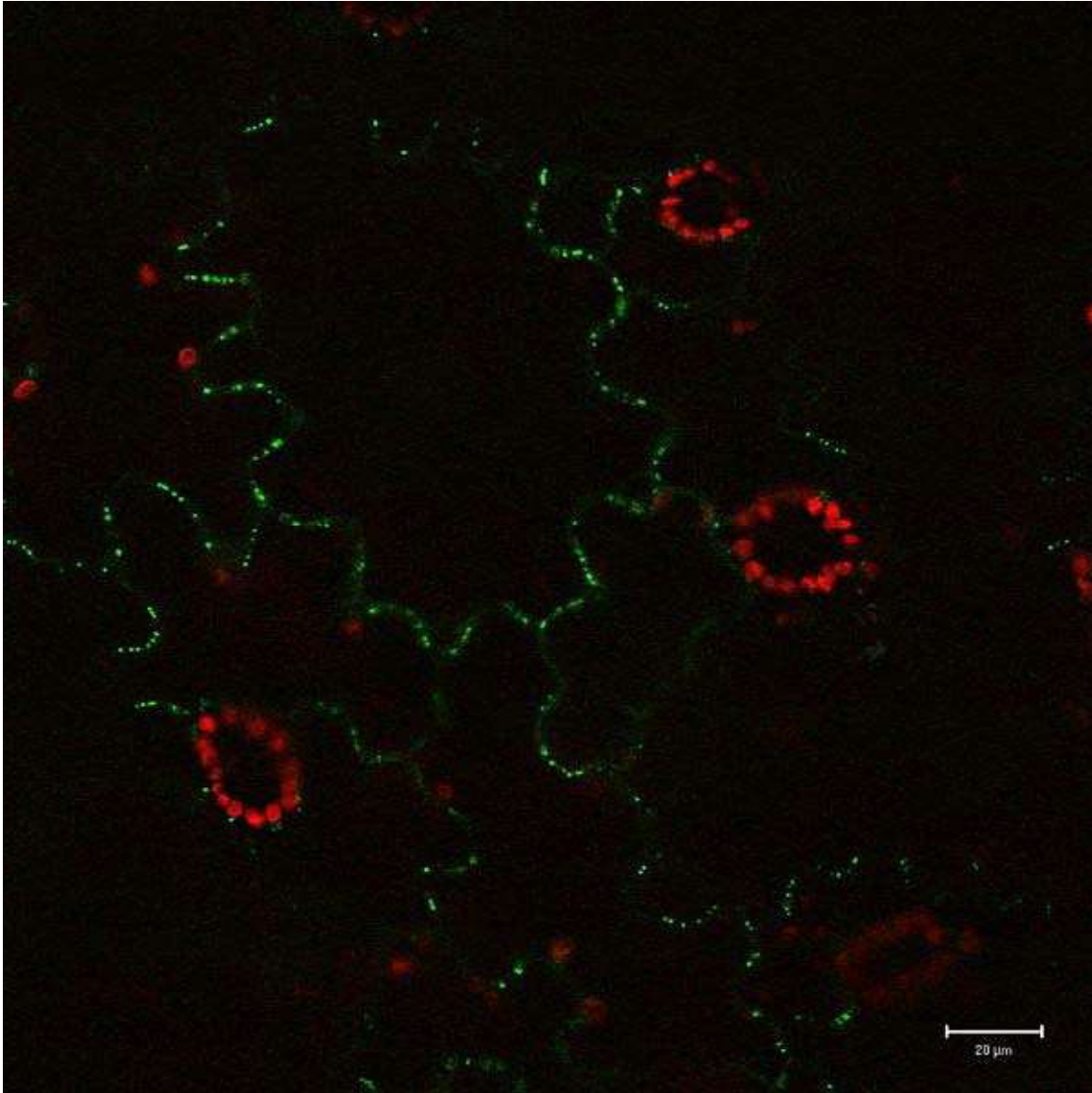
Principle of confocal microscopy.

In a confocal laser scanning microscope, a laser beam passes through a light source aperture and then is focused by an objective lens into a small (ideally diffraction limited) focal volume within or on the surface of a specimen. In biological applications especially, the specimen may be fluorescent. Scattered and reflected laser light as well as any fluorescent light from the illuminated spot is then re-collected by the objective lens. A beam splitter separates off some portion of the light into the detection apparatus, which in fluorescence confocal microscopy will also have a filter that selectively passes the fluorescent wavelengths while blocking the original excitation wavelength. After passing a *pinhole*, the light intensity is detected by a photodetection device (usually a photomultiplier tube (PMT) or avalanche photodiode), transforming the light signal into an electrical one that is recorded by a computer.

The detector aperture obstructs the light that is not coming from the focal point, as shown by the dotted gray line in the image. The out-of-focus light is suppressed: most of the returning light is blocked by the pinhole, which results in sharper images than those from conventional fluorescence microscopy techniques and permits one to obtain images of planes at various depths within the sample (sets of such images are also known as *z stacks*).

The detected light originating from an illuminated volume element within the specimen represents one pixel in the resulting image. As the laser scans over the plane of interest, a whole image is obtained pixel-by-pixel and line-by-line, whereas the brightness of a resulting image pixel corresponds to the relative intensity of detected light. The beam is scanned across the sample in the horizontal plane by using one or more (servo controlled) oscillating mirrors. This scanning method usually has a low reaction latency and the scan speed can be varied. Slower scans provide a better signal-to-noise ratio, resulting in better

contrast and higher resolution. Information can be collected from different focal planes by raising or lowering the microscope stage or objective lens. The computer can generate a three-dimensional picture of a specimen by assembling a stack of these two-dimensional images from successive focal planes.



An example of a GFP fusion protein.

Confocal microscopy provides the capacity for direct, noninvasive, serial optical sectioning of intact, thick, living specimens with a minimum of sample preparation as well as a marginal improvement in lateral resolution. Biological samples are often treated with fluorescent dyes to make selected objects visible. However, the actual dye concentration can be low to minimize the disturbance of biological systems: some instruments can track single fluorescent molecules. Also, transgenic techniques can create

organisms that produce their own fluorescent chimeric molecules (such as a fusion of GFP, green fluorescent protein with the protein of interest).

## **Resolution enhancement**

CLSM is a scanning imaging technique in which the resolution obtained is best explained by comparing it with another scanning technique like that of the scanning electron microscope (SEM). CLSM has the advantage of not requiring a probe to be suspended nanometers from the surface, as in an AFM or STM, for example, where the image is obtained by scanning with a fine tip over a surface. The distance from the objective lens to the surface (called the *working distance*) is typically comparable to that of a conventional optical microscope. It varies with the system optical design, but working distances from hundreds of micrometres to several millimeters are typical.

In CLSM a specimen is illuminated by a point laser source, and each volume element is associated with a discrete scattering or fluorescence intensity. Here, the size of the scanning volume is determined by the spot size (close to diffraction limit) of the optical system because the image of the scanning laser is not an infinitely small point but a three-dimensional diffraction pattern. The size of this diffraction pattern and the focal volume it defines is controlled by the numerical aperture of the system's objective lens and the wavelength of the laser used. This can be seen as the classical resolution limit of conventional optical microscopes using wide-field illumination. However, with confocal microscopy it is even possible to improve on the resolution limit of wide-field illumination techniques because the confocal aperture can be closed down to eliminate higher orders of the diffraction pattern. For example, if the pinhole diameter is set to 1 Airy unit then only the first order of the diffraction pattern makes it through the aperture to the detector while the higher orders are blocked, thus improving resolution at the cost of a slight decrease in brightness. In fluorescence observations, the resolution limit of confocal microscopy is often limited by the signal to noise ratio caused by the small number of photons typically available in fluorescence microscopy. One can compensate for this effect by using more sensitive photodetectors or by increasing the intensity of the illuminating laser point source. Increasing the intensity of illumination laser risks excessive bleaching or other damage to the specimen of interest, especially for experiments in which comparison of fluorescence brightness is required. When imaging tissues which are differentially refractive, such as the spongy mesophyll of plant leaves or other air-space containing tissues, spherical aberrations that impair confocal image quality are often pronounced. Such aberrations however, can be significantly reduced by mounting samples in optically transparent, non-toxic perfluorocarbons such as perfluorodecalin, which readily infiltrates tissues and has a refractive index almost identical to that of water.

## **Uses**

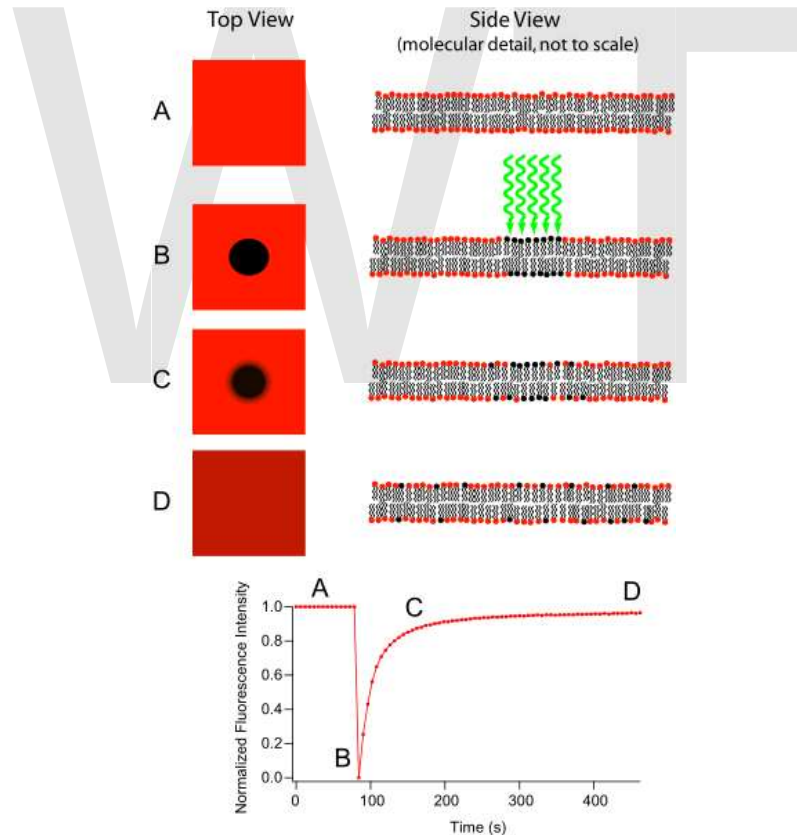
CLSM is widely-used in numerous biological science disciplines, from cell biology and genetics to microbiology and developmental biology.

Clinically, CLSM is used in the evaluation of various eye diseases, and is particularly useful for imaging, qualitative analysis, and quantification of endothelial cells of the cornea. It is used for localizing and identifying the presence of filamentary fungal elements in the corneal stroma in cases of keratomycosis, enabling rapid diagnosis and thereby early institution of definitive therapy. Research into CLSM techniques for endoscopic procedures is also showing promise. In the pharmaceutical industry, it was recommended to follow the manufacturing process of thin film pharmaceutical forms, to control the quality and uniformity of the drug distribution. CLSM is also used as the data retrieval mechanism in some 3D optical data storage systems and has helped determine the age of the Magdalen papyrus.

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## Chapter 10

# Fluorescence Recovery After Photobleaching



Principle of FRAP A) The bilayer is uniformly labeled with a fluorescent tag B) This label is selectively photobleached by a small (~30 micrometre) fast light pulse C) The intensity within this bleached area is monitored as the bleached dye diffuses out and new dye diffuses in D) Eventually uniform intensity is restored

**Fluorescence recovery after photobleaching (FRAP)** denotes an optical technique capable of quantifying the two dimensional lateral diffusion of a molecularly thin film containing fluorescently labeled probes, or to examine single cells. This technique is very useful in biological studies of cell membrane diffusion and protein binding. In addition, surface deposition of a fluorescing phospholipid bilayer (or monolayer) allows the characterization of hydrophilic (or hydrophobic) surfaces in terms of surface structure and free energy. Similar, though less well known, techniques have been developed to investigate the 3-dimensional diffusion and binding of molecules inside the cell; they are also referred to as FRAP.

## ***Experimental Setup***

The basic apparatus comprises an optical microscope, a light source and some fluorescent probe. Fluorescent emission is contingent upon absorption of a specific optical wavelength or color which restricts the choice of lamps. Most commonly, a broad spectrum mercury or xenon source is used in conjunction with a color filter. The technique begins by saving a background image of the sample before photobleaching. Next, the light source is focused onto a small patch of the viewable area either by switching to a higher magnification microscope objective or with laser light of the appropriate wavelength. The fluorophores in this region receive high intensity illumination which causes their fluorescence lifetime to quickly elapse (limited to roughly  $10^5$  photons before extinction). Now the image in the microscope is that of a uniformly fluorescent field with a noticeable dark spot. As Brownian motion proceeds, the still-fluorescing probes will diffuse throughout the sample and replace the non-fluorescent probes in the bleached region. This diffusion proceeds in an ordered fashion, analytically determinable from the diffusion equation. Assuming a Gaussian profile for the bleaching beam, the diffusion constant  $D$  can be simply calculated from:

$$D = \frac{w^2}{4t_{1/2}}$$

where  $w$  is the radius of the beam and  $t_{1/2}$  is the time required for the bleach spot to recover half of its initial integrated intensity.

## ***Applications***

### **Supported Lipid Bilayers**

Originally, the FRAP technique was intended for use as a mean to characterize the mobility of individual lipid molecules within a cell membrane. While providing great utility in this role, current research leans more toward investigation of artificial lipid membranes. Supported by hydrophilic or hydrophobic substrates (to produce lipid bilayers or monolayers respectively) and incorporating membrane proteins, these biomimetic structures are potentially useful as analytical devices for determining the

identity of unknown substances, understanding cellular transduction, and identifying ligand binding sites.

## Protein Binding

This technique is commonly used in conjunction with green fluorescent protein (GFP) fusion proteins, where the studied protein is fused to a GFP. When excited by a specific wavelength of light, the protein will fluoresce. When the protein that is being studied is produced with the GFP, then the fluorescence can be tracked. Photodestroying the GFP, and then watching the repopulation into the bleached area can reveal information about protein interaction partners, organelle continuity and protein trafficking.

If after some time the fluorescence doesn't reach the initial level anymore, then some part of the fluorescence is caused by an immobile fraction (that cannot be replenished by diffusion). Similarly, if the fluorescent proteins bind to static cell receptors, the rate of recovery will be retarded by a factor related to the association and disassociation coefficients of binding. This observation has most recently been exploited to investigate protein binding.

## Applications Outside the Membrane

FRAP can also be used to monitor proteins outside the membrane. After the protein of interest is made fluorescent, generally by expression as a GFP fusion protein, a confocal microscope is used to photobleach and monitor a region of the cytoplasm, mitotic spindle, nucleus, or another cellular structure. The mean fluorescence in the region can then be plotted versus time since the photobleaching, and the resulting curve can yield kinetic coefficients for the protein's binding reactions and/or the protein's diffusion coefficient in the medium where it is being monitored. The analysis is most simple when the curve is dominated by only the diffusional or only the binding components. For a circular bleach spot and diffusion-dominated recovery, the fluorescence is described by the Soumpasis equation and involves modified Bessel functions:

$$f(t) = e^{-h} (I_0(h) + I_1(h))$$

where  $h=r^2/(2*D_f*t)$ ;  $r$ =radius of bleach spot;  $t$ =time;  $D_f$ =diffusion coefficient;  $f(t)$  is the normalized fluorescence (goes to 1 as  $t$  goes to infinity).

For a binding-dominated reaction, in which the diffusion is much faster than the unbinding of the bleached protein and subsequent binding of unbleached protein, it is given by

$$f(t) = 1 - B_{eq}e^{-k_{off}t}$$

where  $B_{eq}$  is the fraction of the protein that is bound to other structures in the photobleached region at equilibrium, and  $k_{off}$  is the dissociation constant for the binding. Sometimes there are multiple binding states in which case there are just more exponential

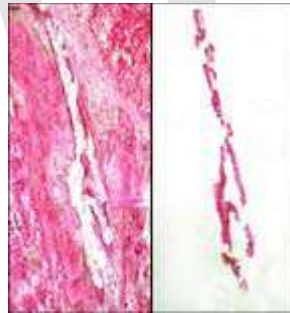
terms of the same form. Many FRAP recoveries are not dominated overwhelmingly by just diffusion or just binding, so their curves are more complex; FRAP recoveries are analyzed in much more detail in Sprague and Pego et al.

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## Chapter 11

# Laser Capture Microdissection and Pulse-Chase Analysis

## Laser capture microdissection



Laser capture micro-dissection transfer of pure breast duct epithelial cells. Left panel shows tissue section with selected cells removed. Right panel shows isolated epithelial cells on transfer film.

**Laser capture microdissection** (LCM), also called Microdissection, Laser MicroDissection (LMD), or Laser-assisted microdissection (LMD or LAM) is a method for isolating specific cells of interest from microscopic regions of tissue/cells/organisms.

### ***Extraction process***

A laser is coupled into a microscope and focuses onto the tissue on the slide. By movement of the laser by optics or the stage the focus follows a trajectory which is predefined by the user. This trajectory, a so called *Element*, is then cut out and separated from the adjacent tissue. After the cutting process, an extraction process has to follow if an extraction process is desired.

Theoretically, there are several ways to extract tissue from a microscope slide with a histopathology sample on it:

- Press a sticky surface onto the sample and tear out. This will extract the desired region, but also bears the chance to carry particles or unwanted tissue on the surface, because an *allround sticky* surface is not selective.
- Melt a plastic membrane onto the sample and tear out. The heat is introduced by an, e.g., red or IR laser onto a membrane stained with an absorbing dye. As this adheres the desired sample onto the membrane, as with any membrane that is put close to the histopathology sample surface, there might be some debris extracted. Another danger is the introduced heat: Some molecules like DNA, RNA, or protein don't allow to be heated too much or at all for the goal of being isolated as purely as possible.
- Transport without contact. There are two different approaches:
  1. Transport simply by gravity using an upright microscope or
  2. Reliable and precise transport by *Laser Pressure Catapult*

## **Procedure**

Under a microscope using a software interface, a tissue section (typically 5-50 micrometres thick) is viewed and individual cells or clusters of cells are identified either manually or in semi-automated or more fully automated ways allowing the imaging and then automatic selection of targets for isolation. Currently five primary isolation/collection technologies exist using a microscope and device for cell isolation. Four of these typically use an ultraviolet pulsed laser (355 nm) for the cutting of the tissues directly or the membranes/film, and sometimes in combination with an IR laser responsible for heating/melting a sticky polymer for cellular adhesion and isolation. IR laser provides a more gentle approach to microdissection.

The various technologies differ in the collection process, possible imaging modalities (Fluorescence microscopy/Bright field microscopy/Differential interference contrast microscopy/Phase contrast microscopy/ etc.) and the types of holders and tissue preparation needed before the imaging and isolation. Most are primarily dedicated Microdissection systems, and some can be used as research microscopes as well, only one technology (#2 here, Leica) uses an upright microscope, limiting some of the sample handling capabilities somewhat esp. for live cell work.

1.) The first technology may first cut around the sample then collect it by a patented "catapulting" technology. The sample can be catapulted from a slide or special culture dish by a defocused U.V laser pulse (generating a photonic force for propelling the material off the slide/dish. This is sometimes called Laser Micro-dissection Pressure Catapulting (LMPC) and the tissue is sent upward (up to several mm) to a microfuge tube cap (or other collector) containing buffer or a specialized tacky material in the tube cap that the tissue/cell/ nucleus/chromosome fragment will then adhere to. This active catapulting process avoids some of the static problems when using membrane-coated slides. This system had historically been offered by PALM Microlaser Technologies and is now a Zeiss product called PALM MicroBeam.

2.) Another closely related LCM process cuts the sample from above and the sample drops via gravity into a capture device below the sample.

3.) When the cells (on a slide or special culture dish) of choice are in the center of the field of view, the operator selects the cells of interest using instrument software. The area to be isolated when a near-IR laser to activate transfer film on a cap placed on the tissue sample, melting the adhesive which then fuses the film with the underlying cells of choice; and/or by activating a UV laser to cut out the cell of interest. The cells are then lifted off the thin tissue section, leaving all unwanted cells behind. The cells of interest are then viewed and documented prior to extraction.

4.) The last UV based technology offers a slight difference to the 3rd technology here by essentially creating a sandwich of sorts with slide>sample>and membrane overlying the sample by the use of a frame slide whose membrane surface is cut by the laser and ultimately picked up from above by a special adhesive cap.

#### NON-LASER BASED MICRODISSECTION/ISOLATION

5.) This last technique cannot really be called Laser Capture Microdissection but is closely related. For lack of a better term is micro-chiseling; by using a piezoelectric driven micro-chisel that vibrates ultrasonic frequencies. This allows for fine etching or chiseling of cells and particles in small areas and for collection via a finely adjustable aspirator to a waiting tube collector. Another related technique for isolating via a micropositioned manipulator is available.

In addition to tissue sections, LCM can be performed on living cells/organisms, cell smears, chromosome preparations, and plant tissue.

### ***Applications***

The laser capture microdissection process does not alter or damage the morphology and chemistry of the sample collected, nor the surrounding cells. For this reason, LCM is a useful method of collecting selected cells for DNA, RNA and/or protein analyses. LCM can be performed on a variety of tissue samples including blood smears, cytologic preparations, cell cultures and aliquots of solid tissue. Frozen and paraffin embedded archival tissue may also be used . On formalin or alcohol fixed paraffin embedded tissues, DNA and RNA retrieval has been successful, but protein analysis is not possible (requires frozen section).

# Pulse-chase analysis

In biochemistry and molecular biology, a **pulse-chase analysis** is a method for examining a cellular process occurring over time by successively exposing the cells to a labeled compound (pulse) and then to the same compound in an unlabeled form (chase). Radioactivity is a commonly used label.

## ***Mechanism***

A selected cell or a group of cells is first exposed to a labeled compound (the pulse) that is to be incorporated into a molecule or system that is studied. The compound then goes through the metabolic pathways and is used in the synthesis of the product studied. For example, a radioactively labeled form of leucine ( $^3\text{H}$ -leucine) can be supplied to a group of pancreatic B cells, which then uses this amino acid in insulin synthesis.

Shortly after introduction of the labeled compound (usually about 5 minutes, but the actual time needed is dependent on the object studied), excess of the same, but unlabeled, substance (the chase) is introduced into the environment. Following the previous example, the production of insulin would continue, but it would no longer contain the radioactive leucine introduced in the pulse phase and would not be visible using radioactive detection methods. However, the movement of the labeled insulin produced during the pulse period could still be tracked within the cell.

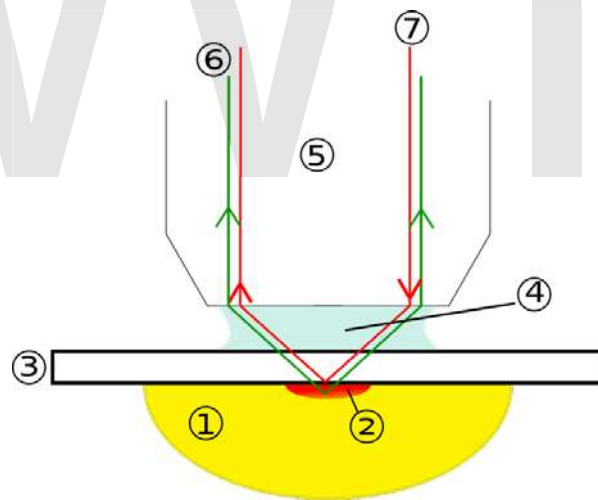
## ***Uses***

This method is useful for determining the activity of certain cells over a prolonged period of time. The method has been used to study protein kinase C, ubiquitin, and many other proteins. The method was also used to prove the existence and function of Okazaki fragments. George Palade used pulse-chase of radioactive amino acids to elicit the secretory pathway.

## Chapter 12

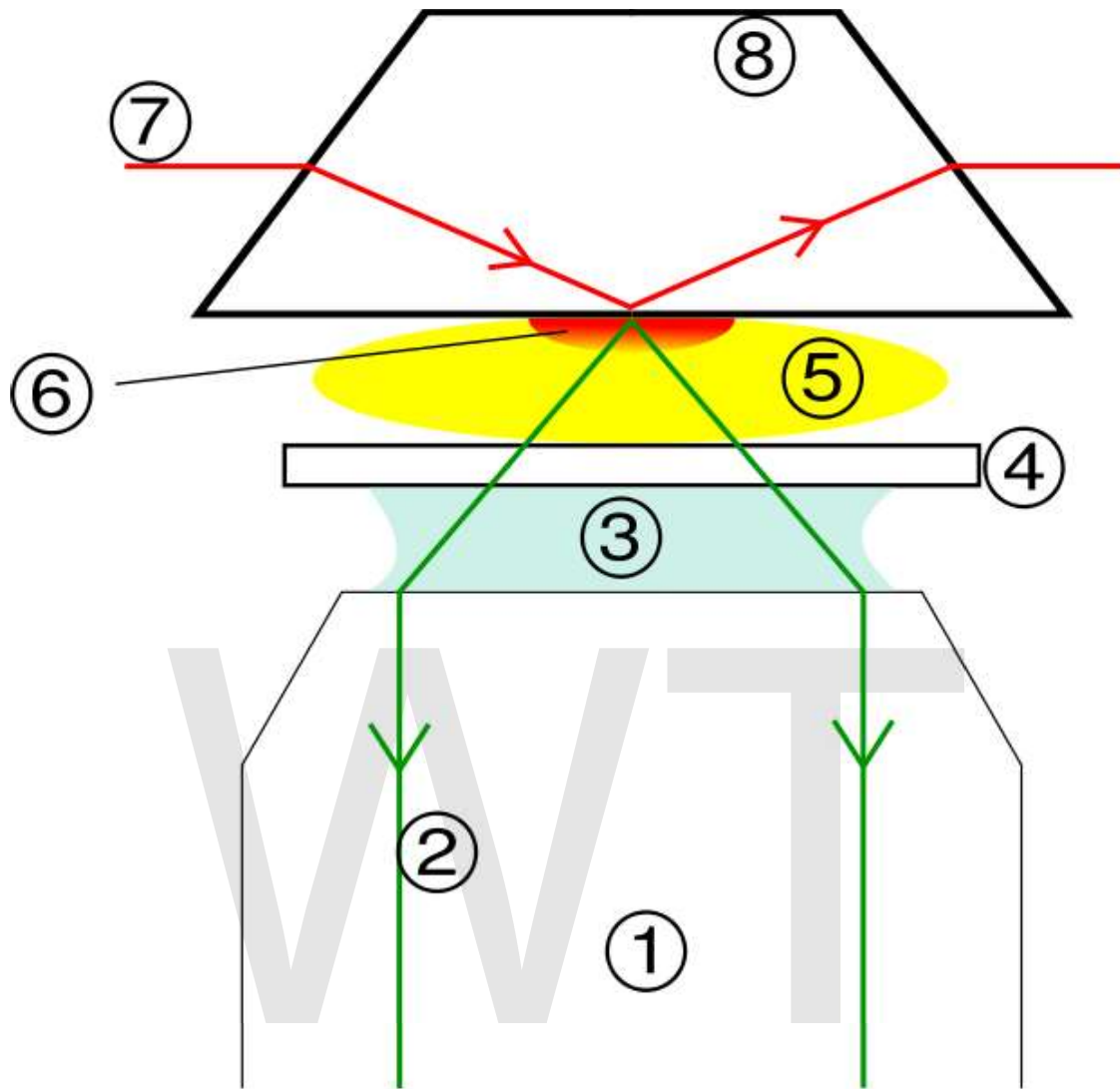
# Total Internal Reflection Fluorescence Microscope and Two-Photon Excitation Microscopy

## Total internal reflection fluorescence microscope



total internal reflection fluorescence microscope (TIRFM) diagram

1. Specimen
2. Evanescent wave range
3. Cover slip
4. Immersion oil
5. Objective
6. Emission beam (signal)
7. Excitation beam



Total Internal Reflection Fluorescence Microscope trans- (TIRFM) diagram

1. Objective
2. Emission beam (signal)
3. Immersion oil
4. Cover slip
5. Specimen
6. Evanescent wave range
7. Excitation beam
8. Quartz prism

A **total internal reflection fluorescence microscope (TIRFM)** is a type of microscope with which a thin region of a specimen, usually less than 200 nm, can be observed.

## **Background**

In cell and molecular biology, a large number of molecular events in cellular surfaces such as cell adhesion, binding of cells by hormones, secretion of neurotransmitters, and membrane dynamics have been studied with conventional fluorescence microscopes. However, fluorophores that are bound to the specimen surface and those in the surrounding medium exist in an equilibrium state. When these molecules are excited and detected with a conventional fluorescence microscope, the resulting fluorescence from those fluorophores bound to the surface is often overwhelmed by the background fluorescence due to the much larger population of non-bound molecules.

## **Solution**

To solve this problem, TIRFM was developed by Daniel Axelrod at the University of Michigan, Ann Arbor in the early 1980s. A TIRFM uses an evanescent wave to selectively illuminate and excite fluorophores in a restricted region of the specimen immediately adjacent to the glass-water interface. The evanescent wave is generated only when the incident light is totally internally reflected at the glass-water interface. The evanescent electromagnetic field decays exponentially from the interface, and thus penetrates to a depth of only approximately 100 nm into the sample medium. Thus the TIRFM enables a selective visualization of surface regions such as the basal plasma membrane (which are about 7.5 nm thick) of cells as shown in the figure above. Note, however, that the region visualised is at least a few hundred nanometers wide, so the cytoplasmic zone immediately beneath the plasma membrane is necessarily visualised in addition to the plasma membrane during TIRF microscopy. The selective visualisation of the plasma membrane renders the features and events on the plasma membrane in living cells with high axial resolution.

TIRF can also be used to observe the fluorescence of a single molecule, making it an important tool of biophysics and quantitative biology.

## **Two-photon excitation microscopy**

**Two-photon excitation microscopy** is a fluorescence imaging technique that allows imaging of living tissue up to a very high depth, that is up to about one millimeter. Being a special variant of the multiphoton fluorescence microscope, it uses red-shifted excitation light which can also excite fluorescent dyes. However for each excitation two photons of the infrared light are absorbed. Using infrared light minimizes scattering in the tissue. Due to the multiphoton absorption the background signal is strongly suppressed. Both effects lead to an increased penetration depth for these microscopes. However, the resolution remains diffraction-limited. Two-photon excitation can be a superior alternative to confocal microscopy due to its deeper tissue penetration, efficient light detection and reduced phototoxicity.

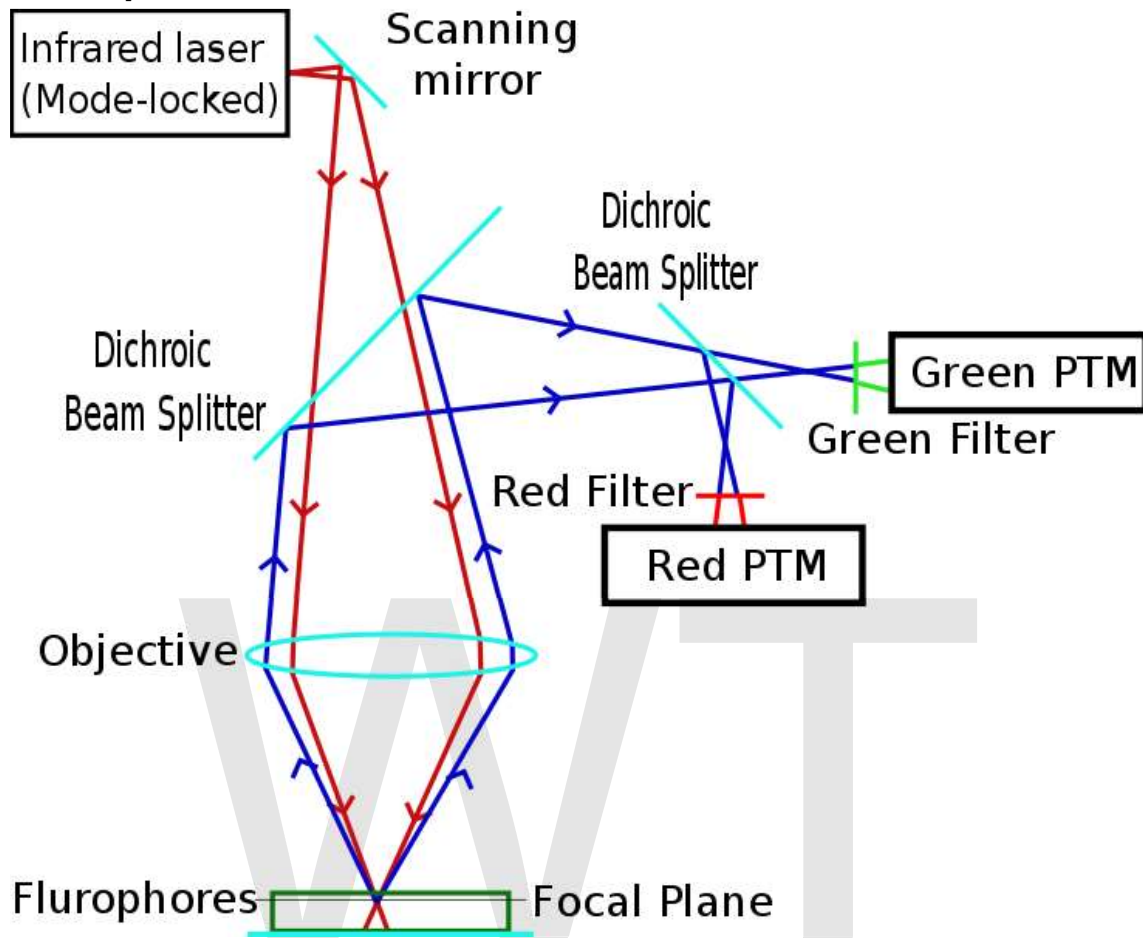
## Concept

Two-photon excitation employs a concept first described by Maria Goeppert-Mayer (1906–1972) in her doctoral dissertation in 1931, and first observed in 1962 in cesium vapor using laser excitation by Isaac Abella.

The concept of two-photon excitation is based on the idea that two photons of comparably lower energy than needed for one photon excitation can also excite a fluorophore in one quantum event. Each photon carries approximately half the energy necessary to excite the molecule. An excitation results in the subsequent emission of a fluorescence photon, typically at a higher energy than either of the two excitatory photons. The probability of the near-simultaneous absorption of two photons is extremely low. Therefore a high flux of excitation photons is typically required, usually a femtosecond laser.

The most commonly used fluorophores have excitation spectra in the 400–500 nm range, whereas the laser used to excite the two-photon fluorescence lies in the ~700–1000 nm (infrared) range. If the fluorophore absorbs two infrared photons simultaneously, it will absorb enough energy to be raised into the excited state. The fluorophore will then emit a single photon with a wavelength that depends on the type of fluorophore used (typically in the visible spectrum). Because two photons are absorbed during the excitation of the fluorophore, the probability for fluorescent emission from the fluorophores increases quadratically with the excitation intensity. Therefore, much more two-photon fluorescence is generated where the laser beam is tightly focused than where it is more diffuse. Effectively, excitation is restricted to the tiny focal volume (~1 femtoliter), resulting in a high degree of rejection of out-of-focus objects. This *localization of excitation* is the key advantage compared to single-photon excitation microscopes, which need to employ additional elements such as pinholes to reject out-of-focus fluorescence. The fluorescence from the sample is then collected by a high-sensitivity detector, such as a photomultiplier tube. This observed light intensity becomes one pixel in the eventual image; the focal point is scanned throughout a desired region of the sample to form all the pixels of the image.

## Development



A diagram of a two-photon microscope

Two-photon microscopy was pioneered by Winfried Denk in the lab of Watt W. Webb at Cornell University. He combined the idea of two-photon absorption with the use of a laser scanner. In two-photon excitation microscopy an infrared laser beam is focused through an objective lens. The Ti-sapphire laser normally used has a pulse width of approximately 100 femtoseconds and a repetition rate of about 80 MHz, allowing the high photon density and flux required for two photons absorption and is tunable across a wide range of wavelengths. Two-photon technology has been patented by Winfried Denk, James Strickler and Watt Webb at Cornell University. Microscope manufacturer Carl Zeiss AG currently holds this patent; Olympus Inc. has licensed it to sell two-photon microscopes.

The use of infrared light to excite fluorophores in light-scattering tissue has added benefits. Longer wavelengths are scattered to a lesser degree than shorter ones, which is a benefit to high-resolution imaging. In addition, these lower-energy photons are less likely to cause damage outside the focal volume. Compared to a confocal microscope, photon detection is much more effective since even scattered photons contribute to the usable signal. There are several caveats to using two-photon microscopy: The pulsed lasers

needed for two-photon excitation are much more expensive than the continuous wave (CW) lasers used in confocal microscopy. The two-photon absorption spectrum of a molecule may vary significantly from its one-photon counterpart. For very thin objects such as isolated cells, single-photon (confocal) microscopes can produce images with higher optical resolution due to their shorter excitation wavelengths. In scattering tissue, on the other hand, the superior optical sectioning and light detection capabilities of the two-photon microscope result in better performance.

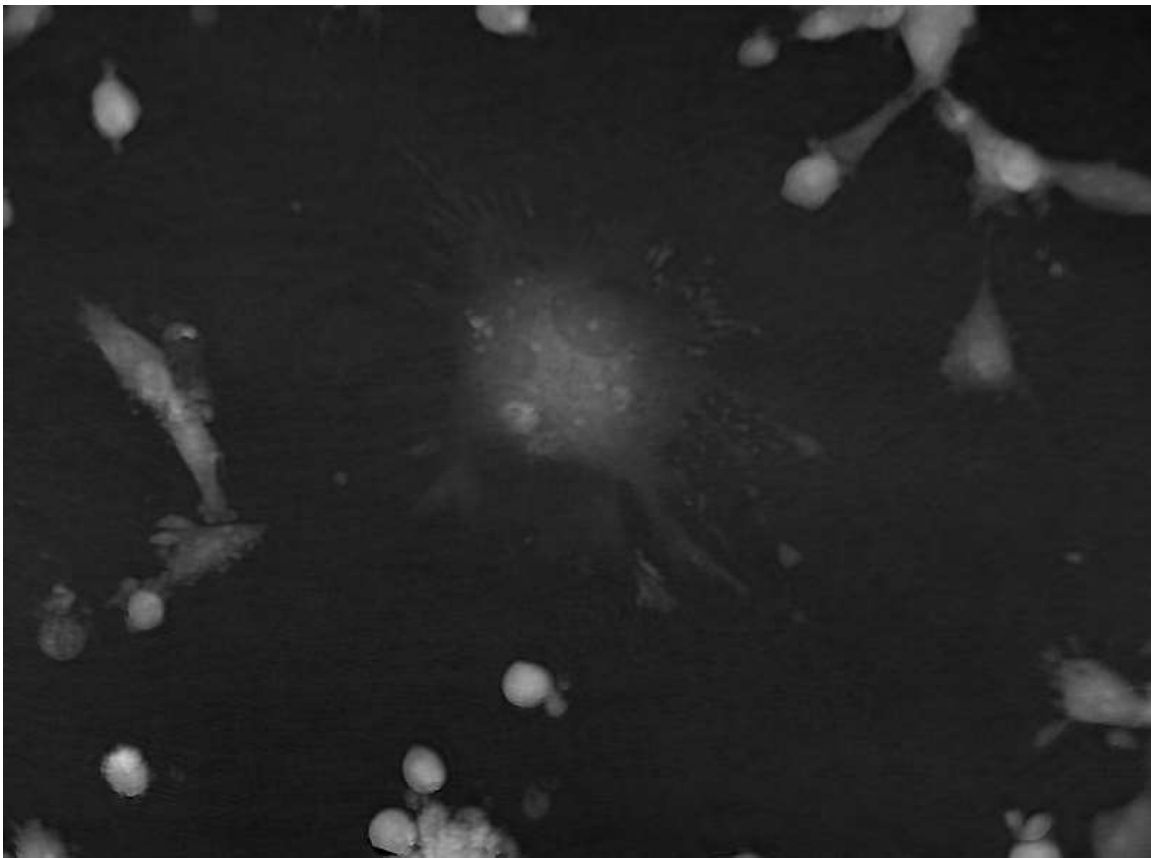
### ***Higher-order excitation***

Simultaneous absorption of three or more photons is also possible, allowing for three-photon or multiphoton excitation microscopy.

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## Chapter 13

# Digital Holographic Microscopy



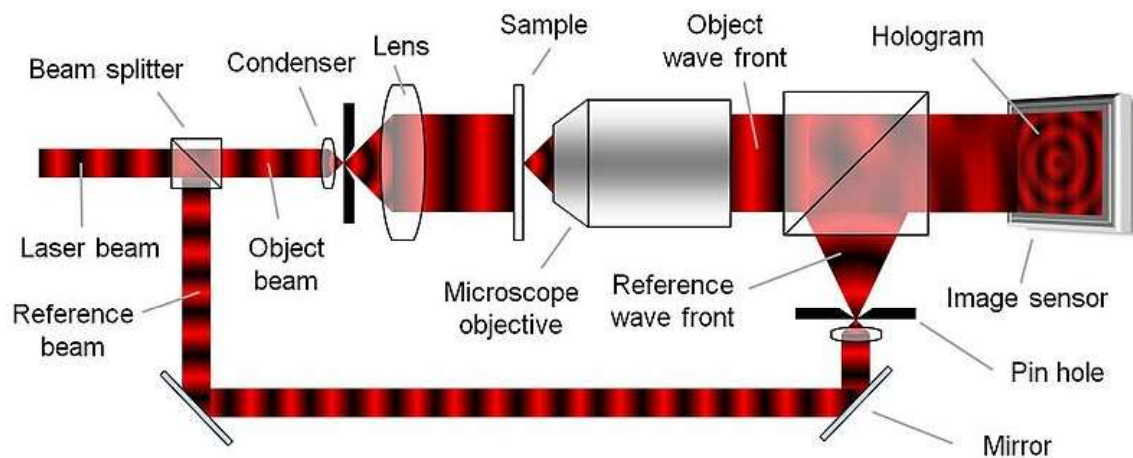
**Figure 1.** DHM phase shift image of cell details.

**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.

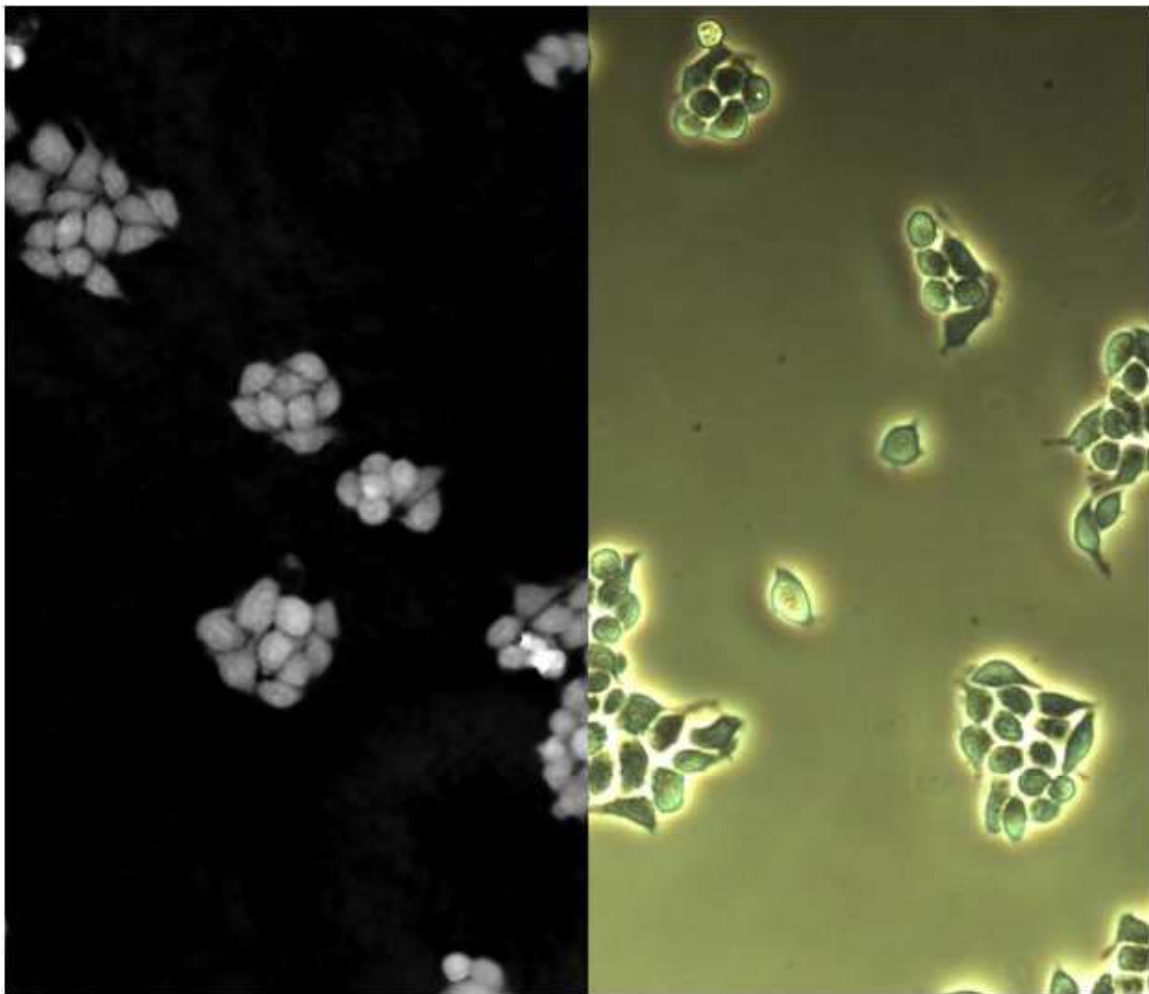
To create the necessary interference pattern, i.e. the hologram, the illumination needs to be a coherent (monochromatic) light source, a laser for example. As can be seen in Figure 2, the laser light is split into an object beam and a reference beam. The expanded object beam illuminates the sample to 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*

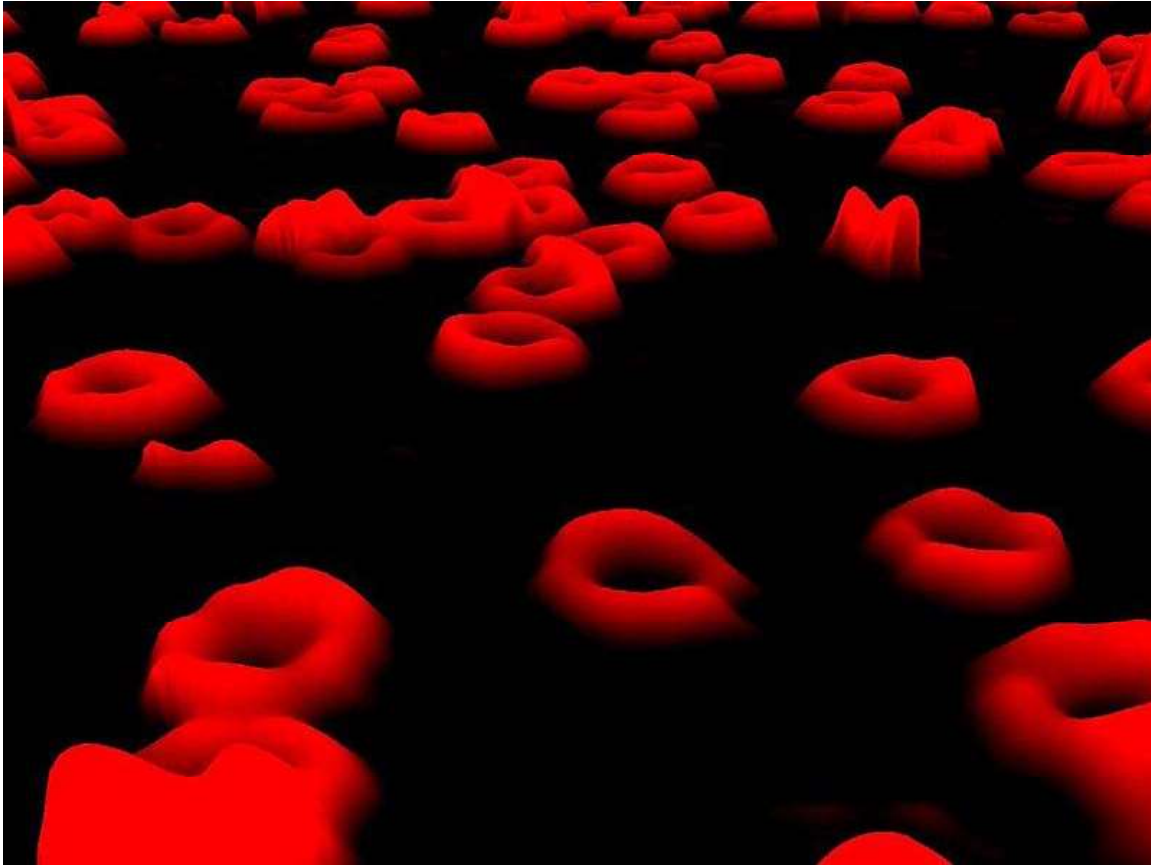
As DHM systems do not have an image forming lens, traditional optical aberrations do not apply to DHM. Optical aberrations are "corrected" by design of the reconstruction algorithm. A reconstruction algorithm that truly models the optical setup will not suffer from optical aberrations.

### *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 14

# Fluorescence Microscope



An upright fluorescence microscope (Olympus BX61) with the fluorescent filter cube turret above the objective lenses, coupled with a digital camera.

A **fluorescence microscope** is an optical microscope used to study properties of organic or inorganic substances using the phenomena of fluorescence and phosphorescence instead of, or in addition to, reflection and absorption. The term "fluorescence microscope" is colloquially synonymous with *epifluorescence microscope* but also refers to microscope designs such as the confocal microscope which also use fluorescence to generate the image.

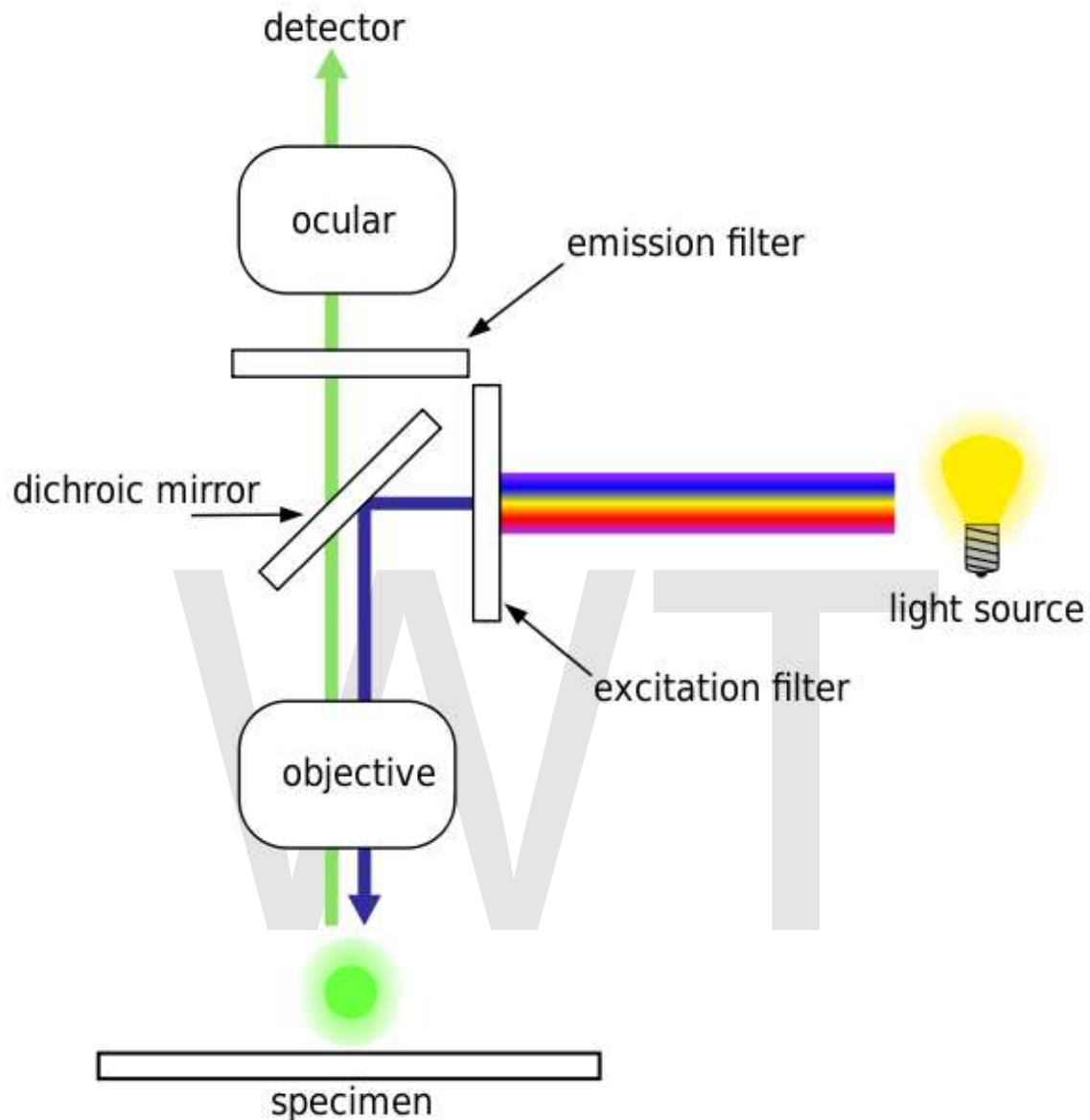
All fluorescence microscopy methods share the same principle. A sample is illuminated with light of a one wavelength which causes fluorescence in the sample. The light emitted by fluorescence, which is at a different, longer, wavelength than the illumination, is then detected through a microscope objective. Two filters are normally used in this technique; an illumination (or excitation) filter which ensures the illumination is near monochromatic and at the correct wavelength, and a second emission (or detection) filter which ensures none of the excitation light source reaches the detector. Fluorescence microscopy takes is a fundamentally different to generating a light microscope image compared to transmitted or reflected white light techniques such as phase contrast and differential interference. These two contrasting optical microscopy methods give very different but complementary data.

### **Principle**

The specimen is illuminated with light of a specific wavelength (or wavelengths) which is absorbed by the fluorophores, causing them to emit light of longer wavelengths (i.e. of a different color than the absorbed light). The illumination light is separated from the much weaker emitted fluorescence through the use of a spectral emission filter. Typical components of a fluorescence microscope are a light source (xenon arc lamp or mercury-vapor lamp), the excitation filter, the dichroic mirror (or dichromatic beamsplitter), and the emission filter. The filters and the dichroic are chosen to match the spectral excitation and emission characteristics of the fluorophore used to label the specimen. In this manner, the distribution of a single fluorophore (color) is imaged at a time. Multi-color images of several types of fluorophores must be composed by combining several single-color images.

Most fluorescence microscopes in use are epifluorescence microscopes (i.e. excitation and observation of the fluorescence are from above (*epi*-) the specimen). These microscopes have become an important part in the field of biology, opening the doors for more advanced microscope designs, such as the confocal microscope and the total internal reflection fluorescence microscope (TIRF).

## Epifluorescence microscopy



Schematic of a fluorescence microscope.

The majority of fluorescence microscopy, especially in the life sciences, is epifluorescence microscopy. The excitatory light is passed from above (or, for inverted microscopes, from below), through the objective lens and then onto the specimen instead of passing it first through the specimen. The fluorescence in the specimen gives rise to emitted light which is focused to the detector by the same objective that is used for the excitation. Since most of the excitatory light is transmitted through the specimen, only reflected excitatory light reaches the objective together with the emitted light and this method therefore gives an improved signal to noise ratio. An additional filter between the objective and the detector can filter out the remaining excitation light from fluorescent

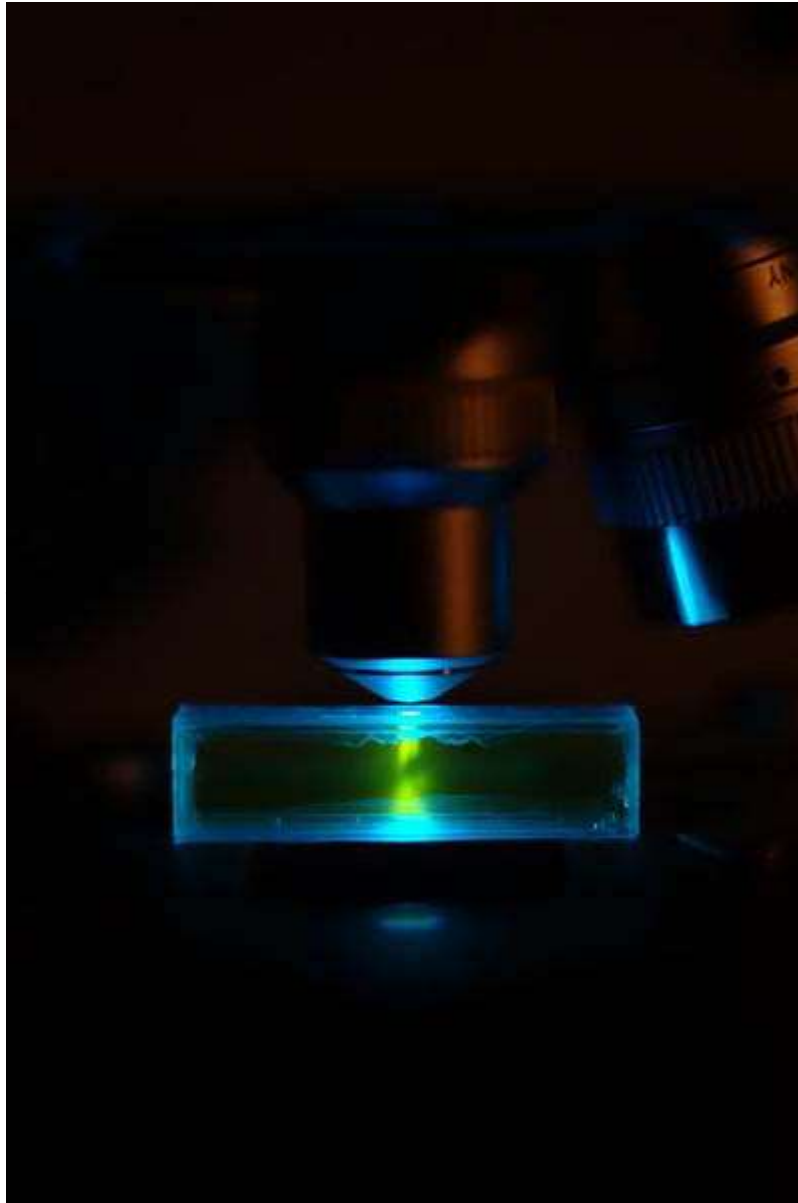
light.

### ***Light sources***

Fluorescence microscopy requires intense, near-monochromatic, illumination which some widespread light sources, like halogen lamps cannot provide. There are two main types of light source used; xenon arc lamp or mercury-vapor lamps with an excitation filter and lasers. Lasers are most widely used for more complex fluorescence microscopy techniques like confocal microscopy and TIRF microscopy while xenon and mercury lamps with an excitation filter are commonly used for widefield epifluorescence microscopes.

WWT

## ***Sample preparation***



A sample of herring sperm stained with SYBR green in a cuvette illuminated by blue light in an epifluorescence microscope. The SYBR green in the sample binds to the herring sperm DNA and, once bound, fluoresces giving off green light when illuminated by blue light.

In order for a sample to be suitable for fluorescence microscopy it must be fluorescent. There are several methods of creating a fluorescent sample; the main techniques are labelling with fluorescent stains or, in the case of biological samples, expression of a fluorescent protein. Alternatively the intrinsic fluorescence of a sample (i.e. autofluorescence) can be used. In the life sciences fluorescence microscopy is a powerful tool which allows the specific and sensitive staining of a specimen in order to detect the

distribution of proteins or other molecules of interest. As a result there is a diverse range of techniques for fluorescent staining of biological samples.

## **Biological fluorescent stains**

Many fluorescent stains have been designed for a range of biological molecules. Some of these are small molecules which are intrinsically fluorescent and bind a biological molecule of interest. Major examples of these are nucleic acid stains like DAPI and Hoescht which bind the minor groove of DNA, thus labelling the nuclei of cells. Others are drugs or toxins which bind specific cellular structures and have been derivitised with a fluorescent reporter. A major example of this class of fluorescent stain is fluorescently labelled-phalloidin which is used to stain actin fibres in mammalian cells.

There are many fluorescent reported molecules, called fluorophores such as fluorescein and DyLight 488, which can be chemically linked to a different molecule which binds the target of interest within the sample.

## **Immunofluorescence**

Immunofluorescence is an antibody based technique which uses the highly specific binding of an antibody to its antigen in order to label specific proteins or other molecules within the cell. A sample is treated with a primary antibody specific for the molecule of interest. A fluorophore can be directly conjugated to the primary antibody. Alternatively a secondary antibody, conjugated to a fluorophore, which binds specifically to the first antibody can be used. For example a primary antibody raised in a mouse which recognises tubulin combined with a secondary anti-mouse antibody derivitised with a fluorophore could be used to label microtubules in a cell.

## **Fluorescent proteins**

The modern understanding of genetics and the techniques available for modifying DNA allows scientists to genetically modify proteins to also carry a fluorescent protein reporter. In biological samples this allows a scientist to directly make a protein of interest fluorescent. The protein location can then be directly tracked, including in live cells.

## ***Limitations***

Fluorophores lose their ability to fluoresce as they are illuminated in a process called photobleaching. Photobleaching occurs as the fluorescent molecules accumulate chemical damage from the electrons excited during fluorescence. Photobleaching can severely limit the time over which a sample can be observed by fluorescent microscopy. Several techniques exist to reduce photobleaching such as the use of more robust fluorophores, by minimizing illumination, or by using photoprotective or scavenger chemicals.

Fluorescence microscopy with fluorescent reporter proteins has enabled analysis of live cells by fluorescence microscopy, however cells are susceptible to phototoxicity,

particularly with short wavelength light. Furthermore fluorescent molecules have a tendency to generate reactive chemical species when under illumination which enhances the phototoxic effect.

Unlike transmitted and reflected light microscopy techniques fluorescence microscopy only allows observation of the specific structures which have been fluorescently labeled. For example observing a tissue sample prepared with a fluorescent DNA stain by fluorescence microscopy only reveals the organisation of the DNA within the cells and reveals nothing else about the cell morphologies.

### ***Improvements and sub-diffraction techniques***

The wave nature of light limits the size of the spot to which light can be focused due to the diffraction limit. This limitation was described in the 19th century by Ernst Abbe and limits an optical microscope's resolution to approximately half of the wavelength of the light used. Fluorescence microscopy is central to many techniques which aim to reach past this limit by specialised optical configurations.

Several improvements in microscopy techniques have been invented in the 20th century and have resulted in increased resolution and contrast to some extent. However they did not overcome the diffraction limit. In 1978 first theoretical ideas have been developed to break this barrier by using a 4Pi microscope as a confocal laser scanning fluorescence microscope where the light is focused ideally from all sides to a common focus which is used to scan the object by 'point-by-point' excitation combined with 'point-by-point' detection. However, the first experimental demonstration of the 4pi microscope took place in 1994. 4Pi microscopy maximizes the amount of available focusing directions by using two opposing objective lenses or Multi-photon microscopy using redshifted light and multi-photon excitation.

The first technique to really achieve a sub-diffraction resolution was STED microscopy, proposed in 1994. This method and all techniques following the RESOLFT concept rely on a strong non-linear interaction between light and fluorescing molecules. The molecules are driven strongly between distinguishable molecular states at each specific location, so that finally light can be emitted at only a small fraction of space, hence an increased resolution.

As well in the 1990s another super resolution microscopy method based on wide field microscopy has been developed. Substantially improved size resolution of cellular nanostructures stained with a fluorescent marker was achieved by development of SPDM localization microscopy and the structured laser illumination (spatially modulated illumination, SMI). Combining the principle of SPDM with SMI resulted in the development of the Vertico SMI microscope. Single molecule detection of normal blinking fluorescent dyes like GFP can be achieved by using a further development of SPDM the so-called SPDMphymod technology which makes it possible to detect and count two different fluorescent molecule types at the molecular level (this technology is referred to as 2CLM, 2 Color Localization Microscopy).

Alternatively, the advent of photoactivated localization microscopy could achieve similar results by relying on blinking or switching of single molecules, where the fraction of fluorescing molecules is very small at each time. This stochastic response of molecules on the applied light corresponds also to a highly nonlinear interaction, leading to subdiffraction resolution.

### ***Fluorescence microscope gallery***

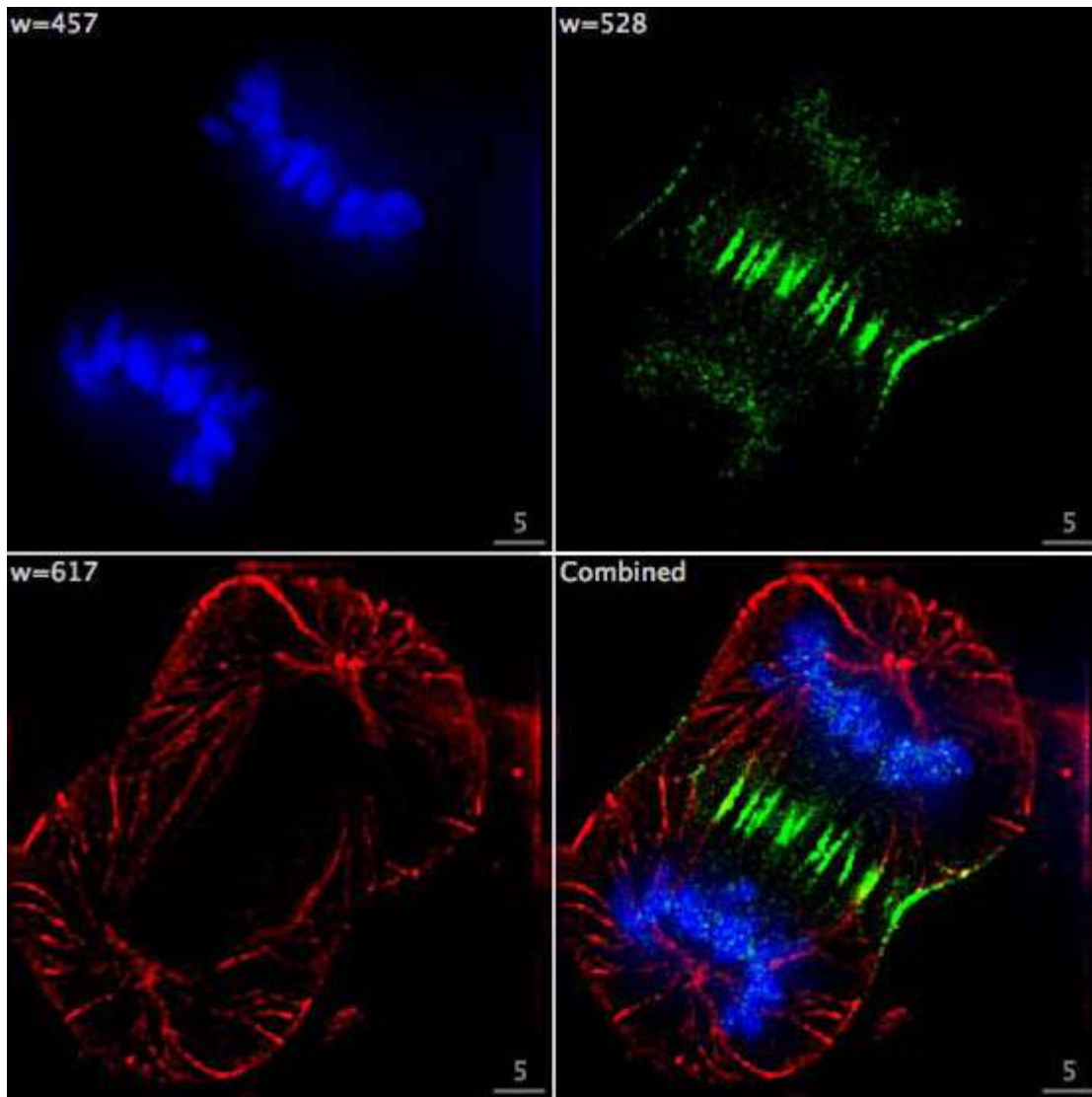


An inverted fluorescence microscope (AMG EVOS fl) with an LCD display instead of oculars for fluorescence microscopy.

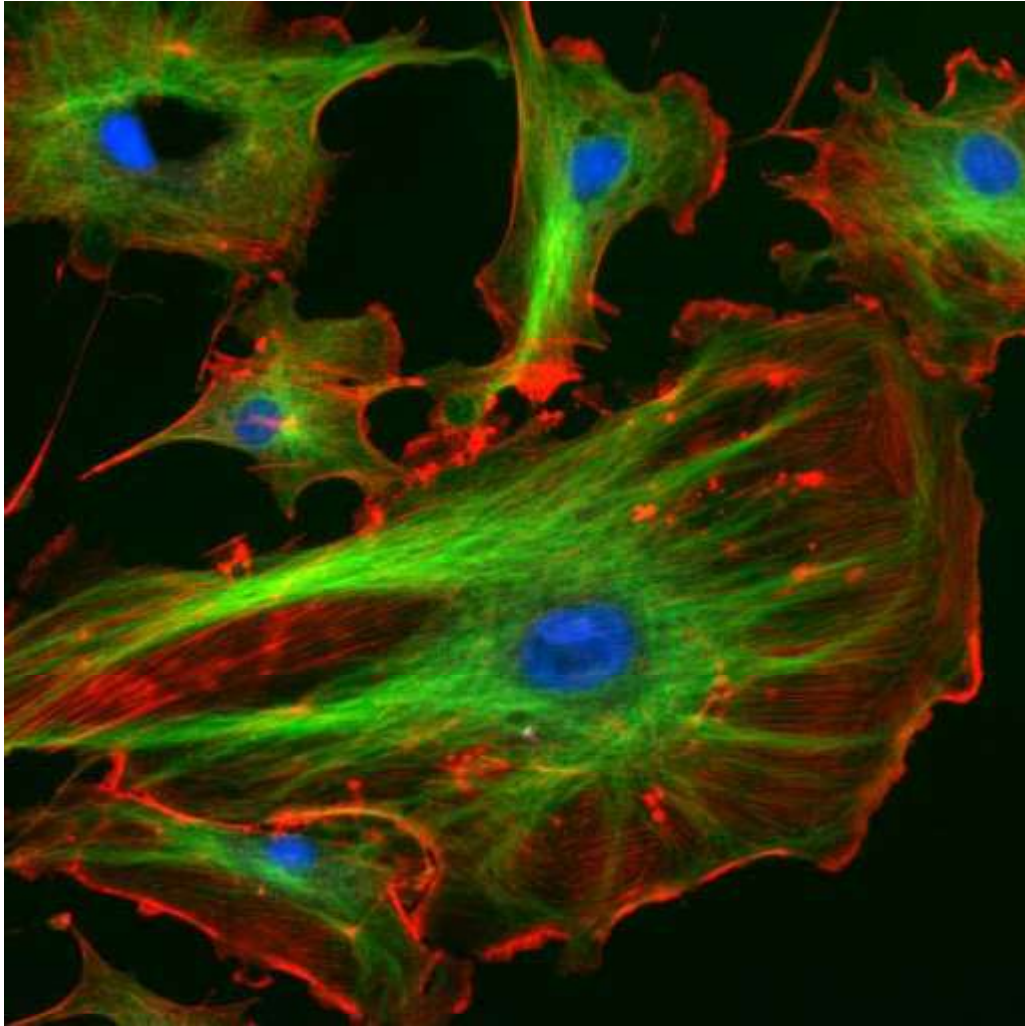


An inverted fluorescence microscope (Nikon TE2000) with the fluorescent filter cube turret below the stage. Note the orange plate that allows the user to look at a sample while protecting their eyes from the UV light.

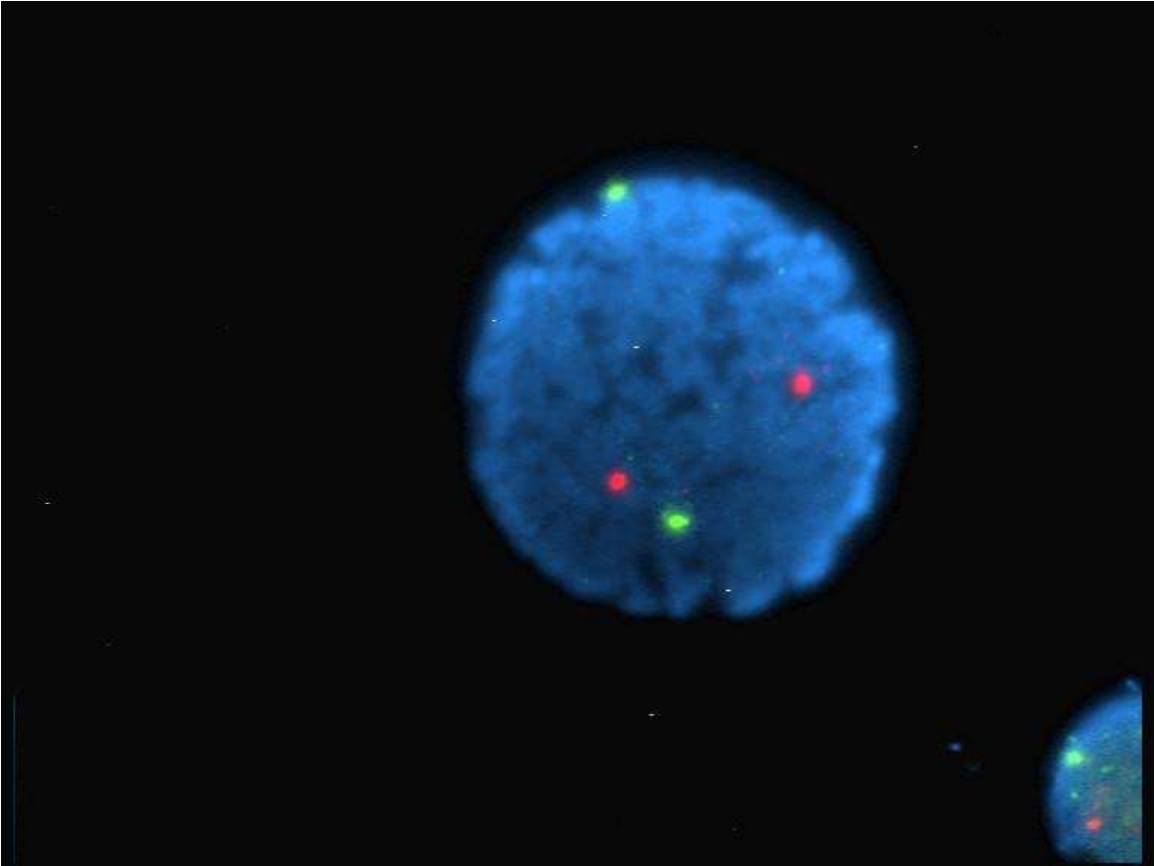
## Fluorescence micrograph gallery



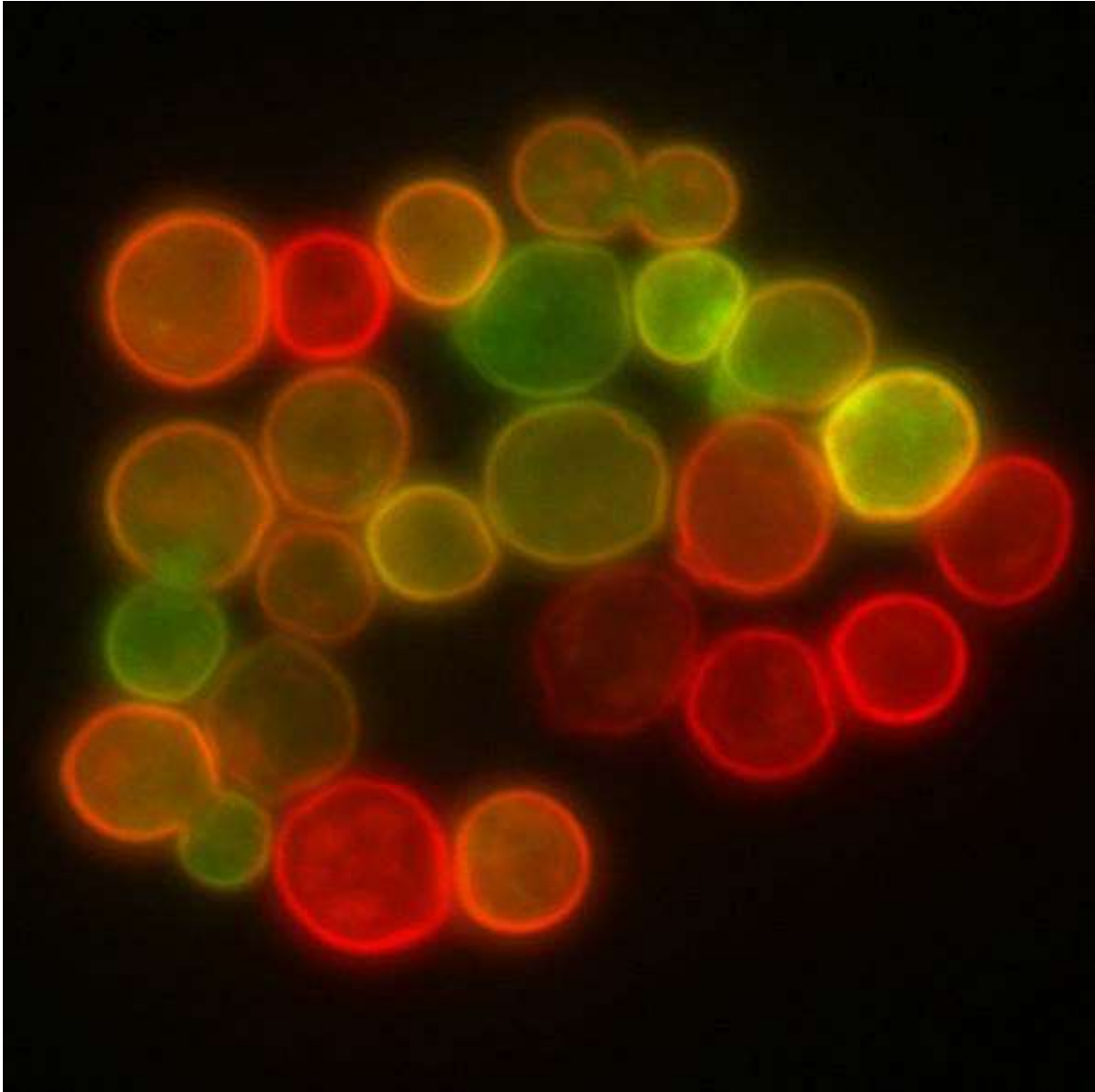
Epifluorescent imaging of the three components in a dividing human cancer cell. DNA is stained blue, a protein called INCENP is green, and the microtubules are red. Each fluorophore is imaged separately using a different combination of excitation and emission filters, and the images are captured sequentially using a digital CCD camera, then overlaid to give a complete image.



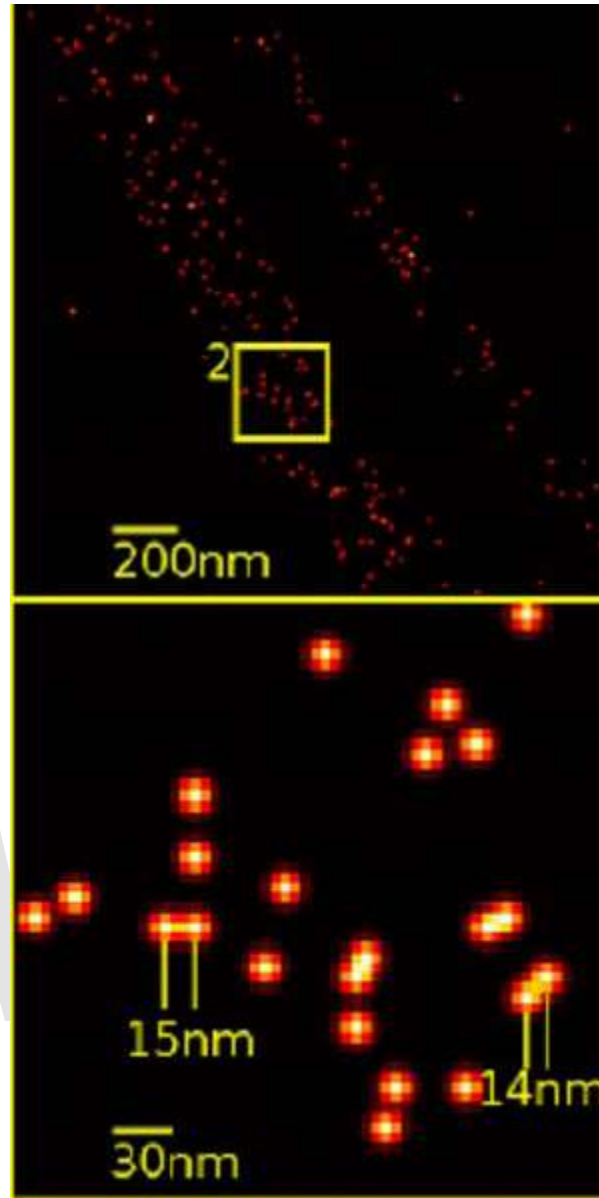
Endothelial cells under the microscope. Nuclei are stained blue with DAPI, microtubules are marked green by an antibody bound to FITC and actin filaments are labeled red with phalloidin bound to TRITC. Bovine pulmonary artery endothelial (BPAE) cells



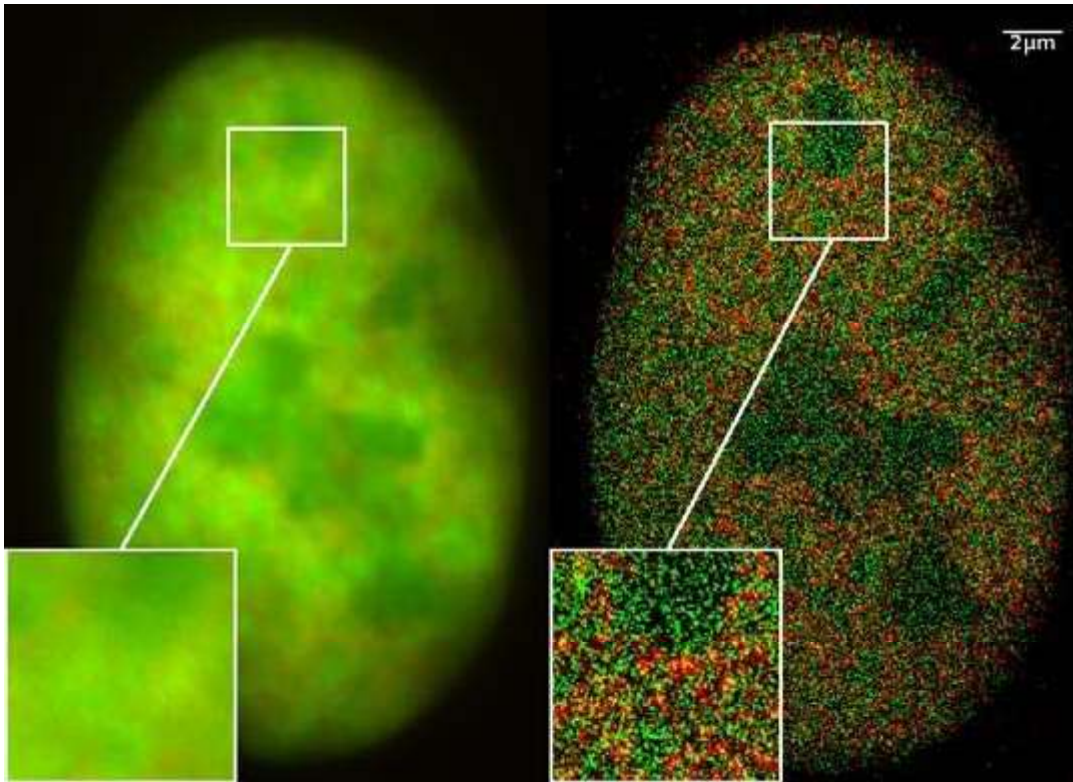
Human lymphocyte nucleus stained with DAPI with chromosome 13 (green) and 21 (red) centromere probes hybridized (Fluorescent in situ hybridization (FISH))



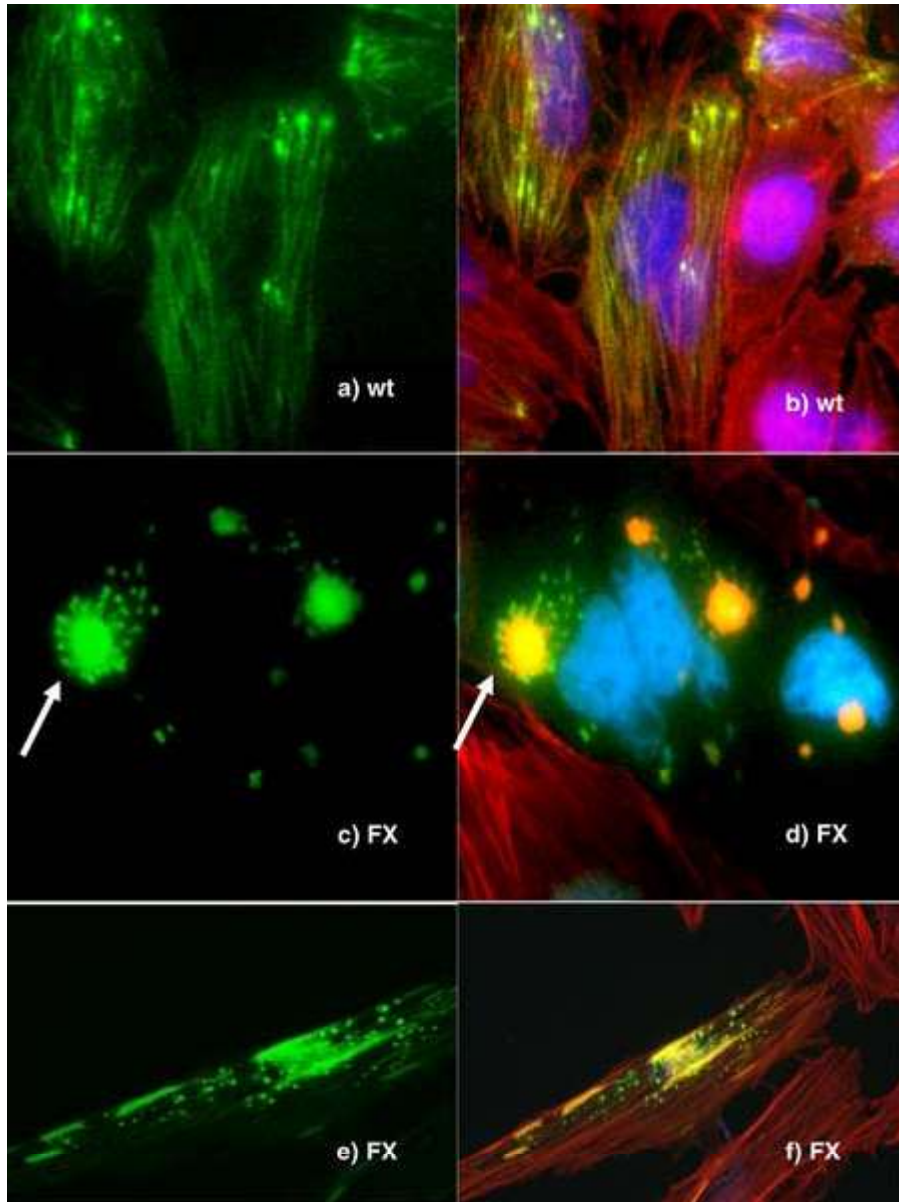
Yeast cell membrane visualized by some membrane proteins fused with RFP and GFP fluorescent markers. Imposition of light from both of markers results in yellow color.



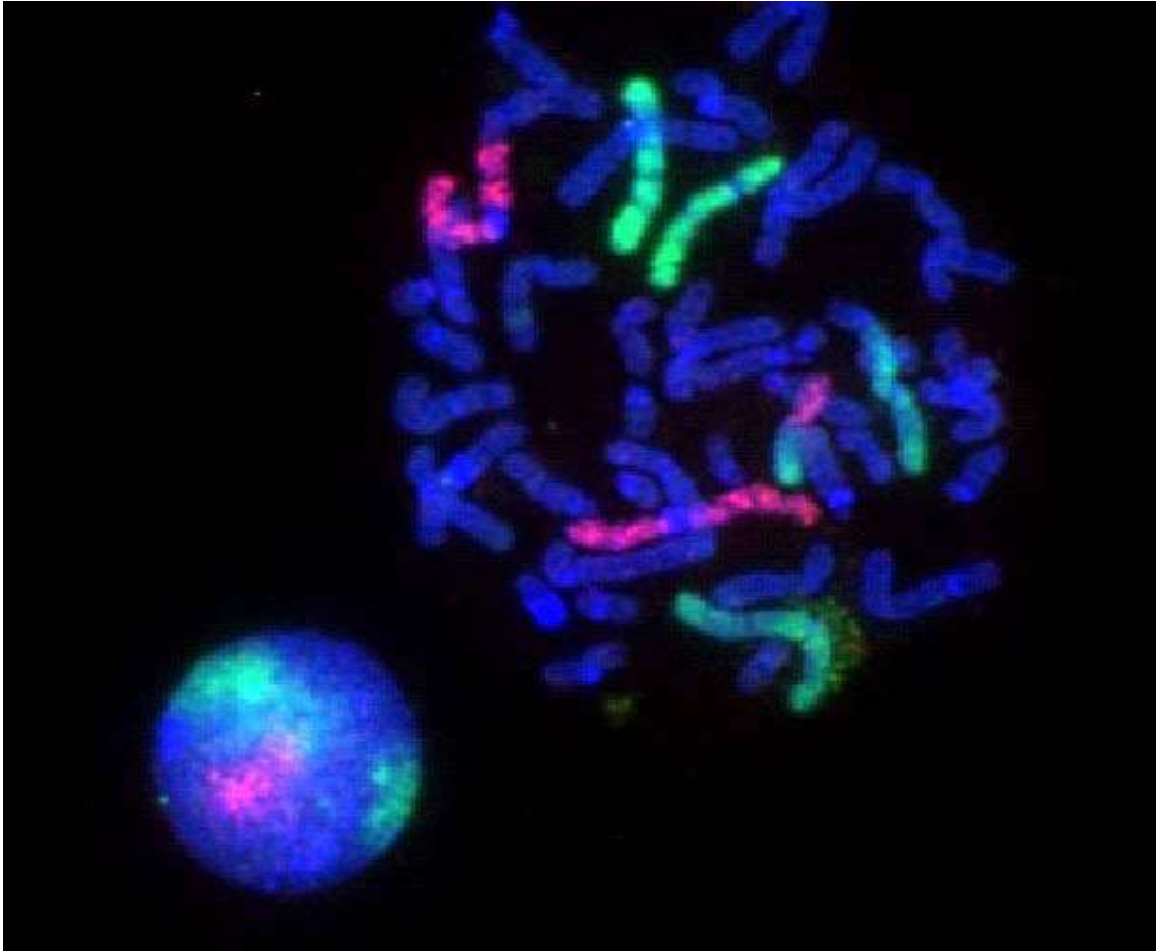
Super Resolution Microscopy: Single YFP molecule detection in a human cancer cell.  
Typical distance measurements in the 15 nm range (5 nm standard deviation) measured with  
a Vertico-SMI/SPDMphymod microscope



Super Resolution Microscopy: Co-localization microscopy (2CLM) with GFP and RFP fusion proteins (nucleus of a bone cancer cell) 120.000 localized molecules in a wide-field area ( $470 \mu\text{m}^2$ ) measured with a Vertico-SMI/SPDMphymod microscope



Fluorescence microscopy of DNA Expression in the Human Wild-Type and P239S Mutant Palladin.



Fluorescence microscopy images of sun flares pathology in a blood cell showing the affected areas in red.

## Chapter 15

# Förster Resonance Energy Transfer



Fluorescently-labeled guanosine 5'-triphosphate hydrolase ARF reveals the protein's localization in the Golgi apparatus of a living macrophage. FRET studies revealed ARF activation in the Golgi and in the formation of phagosomes.

**Förster resonance energy transfer** (abbreviated **FRET**), also known as **fluorescence resonance energy transfer**, **resonance energy transfer (RET)** or **electronic energy transfer (EET)**, is a mechanism describing energy transfer between two chromophores.

A donor chromophore, initially in its electronic excited state, may transfer energy to an acceptor chromophore (in proximity, typically less than 10 nm) through nonradiative dipole–dipole coupling. This mechanism is termed "Förster resonance energy transfer" and is named after the German scientist Theodor Förster. When both chromophores are fluorescent, the term "fluorescence resonance energy transfer" is often used instead, although the energy is not actually transferred by fluorescence. In order to avoid an erroneous interpretation of the phenomenon that (even when occurring between two fluorescent chromophores) is always a nonradiative transfer of energy, the name "Förster resonance energy transfer" is preferred to "fluorescence resonance energy transfer" – the latter enjoys common usage in scientific literature. FRET is analogous to near field communication, in that the radius of interaction is much smaller than the wavelength of light emitted. In the near field region, the excited chromophore emits a virtual photon that is instantly absorbed by a receiving chromophore. These virtual photons are undetectable, since their existence violates the conservation of energy and momentum, and hence FRET is known as a *radiationless* mechanism. From quantum electrodynamical calculations, it is determined that radiationless (FRET) and radiative energy transfer are the short- and long-range asymptotes of a single unified mechanism.

### **Theoretical basis**

The FRET efficiency ( $E$ ) is the quantum yield of the energy transfer transition, *i.e.* the fraction of energy transfer event occurring per donor excitation event:

$$E = \frac{k_{ET}}{k_f + k_{ET} + \sum k_i}$$

where  $k_{ET}$  is the rate of energy transfer,  $k_f$  the radiative decay rate and the  $k_i$  are the rate constants of any other de-excitation pathway.

The FRET efficiency depends on many parameters that can be grouped as follows:

- The distance between the donor and the acceptor
- The spectral overlap of the donor emission spectrum and the acceptor absorption spectrum.
- The relative orientation of the donor emission dipole moment and the acceptor absorption dipole moment.

$E$  depends on the donor-to-acceptor separation distance  $r$  with an inverse 6th power law due to the dipole-dipole coupling mechanism:

$$E = \frac{1}{1 + (r/R_0)^6}$$

with  $R_0$  being the Förster distance of this pair of donor and acceptor, *i.e.* the distance at which the energy transfer efficiency is 50%. The Förster distance depends on the overlap

integral of the donor emission spectrum with the acceptor absorption spectrum and their mutual molecular orientation as expressed by the following equation:

$$R_0^6 = \frac{9000 Q_0 (\ln 10) \kappa^2 J}{128 \pi^5 n^4 N_A}$$

where  $Q_0$  is the fluorescence quantum yield of the donor in the absence of the acceptor,  $\kappa^2$  is the dipole orientation factor,  $n$  is the refractive index of the medium,  $N_A$  is Avogadro's number, and  $J$  is the spectral overlap integral calculated as

$$J = \int f_D(\lambda) \epsilon_A(\lambda) \lambda^4 d\lambda$$

where  $f_D$  is the normalized donor emission spectrum, and  $\epsilon_A$  is the acceptor molar extinction coefficient.  $\kappa^2 = 2/3$  is often assumed. This value is obtained when both dyes are freely rotating and can be considered to be isotropically oriented during the excited state lifetime. If either dye is fixed or not free to rotate, then  $\kappa^2 = 2/3$  will not be a valid assumption. In most cases, however, even modest reorientation of the dyes results in enough orientational averaging that  $\kappa^2 = 2/3$  does not result in a large error in the estimated energy transfer distance due to the sixth power dependence of  $R_0$  on  $\kappa^2$ . Even when  $\kappa^2$  is quite different from  $2/3$  the error can be associated with a shift in  $R_0$  and thus determinations of changes in relative distance for a particular system are still valid. Fluorescent proteins do not reorient on a timescale that is faster than their fluorescence lifetime. In this case  $0 \leq \kappa^2 \leq 4$ .

The FRET efficiency relates to the quantum yield and the fluorescence lifetime of the donor molecule as follows:

$$E = 1 - \tau'_D / \tau_D$$

where  $\tau'_D$  and  $\tau_D$  are the donor fluorescence lifetimes in the presence and absence of an acceptor, respectively, or as

$$E = 1 - F'_D / F_D$$

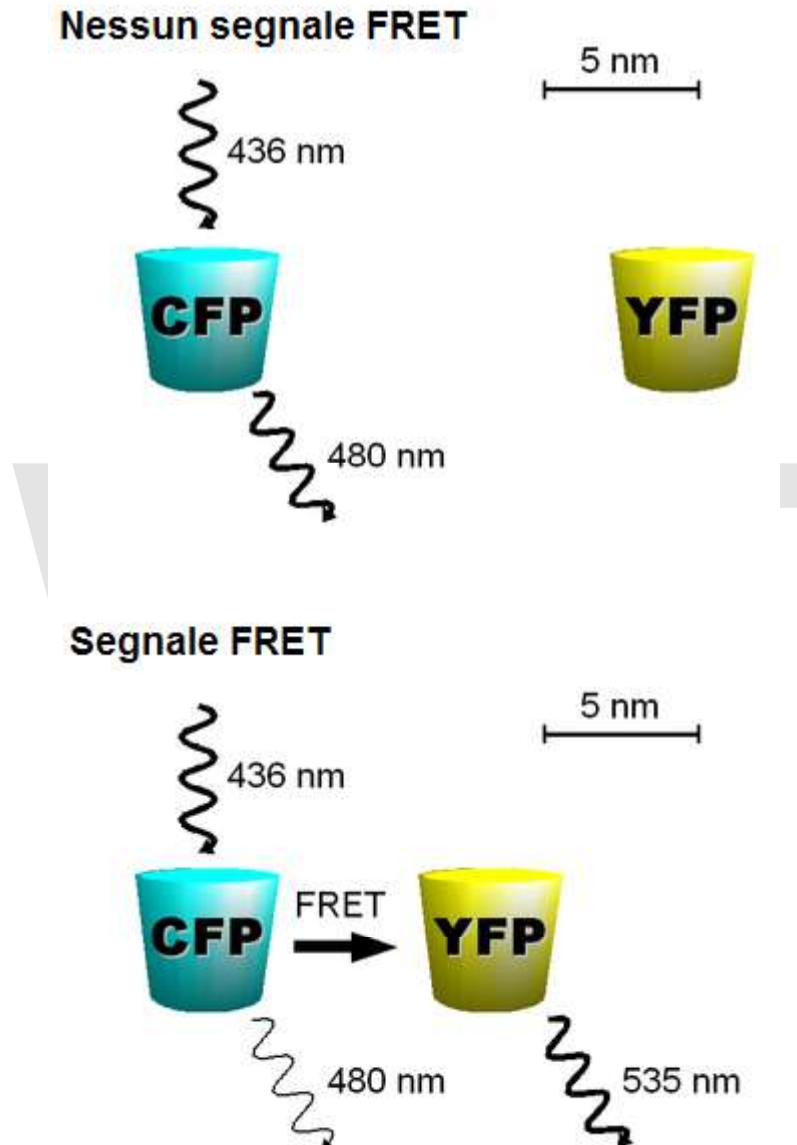
where  $F'_D$  and  $F_D$  are the donor fluorescence intensities with and without an acceptor, respectively.

### ***Experimental Confirmation of the Förster resonance energy transfer theory***

The inverse sixth power distance dependence of Förster resonance energy transfer was experimentally confirmed by Stryer and Haugland using a donor and an acceptor separated on an oligoproline helix. Haugland, Yguerabide and Stryer also experimentally

demonstrated the theoretical dependence of Förster resonance energy transfer on the overlap integral by using a fused indolosteroid as a donor and a ketone as an acceptor.

## Methods



Example of FRET between CFP and YFP (Wavelength vs. Absorption): a fusion protein containing CFP and YFP excited at 440nm wavelength. The fluorescent emission peak of CFP overlaps the excitation peak of YFP. Because the two proteins are adjacent to each other, the energy transfer is significant—a large proportion of the energy from CFP is transferred to YFP and creates a much larger YFP emission peak.

In fluorescence microscopy, fluorescence confocal laser scanning microscopy, as well as in molecular biology, FRET is a useful tool to quantify molecular dynamics in biophysics and biochemistry, such as protein-protein interactions, protein-DNA interactions, and protein conformational changes. For monitoring the complex formation between two

molecules, one of them is labeled with a donor and the other with an acceptor, and these fluorophore-labeled molecules are mixed. When they are dissociated, the donor emission is detected upon the donor excitation. On the other hand, when the donor and acceptor are in proximity (1-10 nm) due to the interaction of the two molecules, the acceptor emission is predominantly observed because of the intermolecular FRET from the donor to the acceptor. For monitoring protein conformational changes, the target protein is labeled with a donor and an acceptor at two loci. When a twist or bend of the protein brings the change in the distance or relative orientation of the donor and acceptor, FRET change is observed. If a molecular interaction or a protein conformational change is dependent on ligand binding, this FRET technique is applicable to fluorescent indicators for the ligand detection.

FRET studies are scalable: the extent of energy transfer is often quantified from the milliliter scale of cuvette-based experiments to the femtoliter scale of microscopy-based experiments. This quantification can be based directly (sensitized emission method) on detecting two emission channels under two different excitation conditions (primarily donor and primarily acceptor). However, for robustness reasons, FRET quantification is most often based on measuring changes in fluorescence intensity or fluorescence lifetime upon changing the experimental conditions (e.g. a microscope image of donor emission is taken with the acceptor being present. The acceptor is then bleached, such that it is incapable of accepting energy transfer and another donor emission image is acquired. A pixel-based quantification using the second equation in the theory section above is then possible.) An alternative way of temporarily deactivating the acceptor is based on its fluorescence saturation. Exploiting polarisation characteristics of light, a FRET quantification is also possible with only a single camera exposure.

### **CFP-YFP pairs**

The most popular FRET pair for biological use is a cyan fluorescent protein (**CFP**) - yellow fluorescent protein (**YFP**) pair. Both are color variants of green fluorescent protein (**GFP**). While labeling with organic fluorescent dyes requires troublesome processes of purification, chemical modification, and intracellular injection of a host protein, GFP variants can be easily attached to a host protein by genetic engineering. By virtue of GFP variants, the use of FRET techniques for biological research is becoming more and more popular.

### **BRET**

A limitation of FRET is the requirement for external illumination to initiate the fluorescence transfer, which can lead to background noise in the results from direct excitation of the acceptor or to photobleaching. To avoid this drawback, Bioluminescence Resonance Energy Transfer (or **BRET**) has been developed. This technique uses a bioluminescent luciferase (typically the luciferase from *Renilla reniformis*) rather than CFP to produce an initial photon emission compatible with YFP.

FRET and BRET are also the common tools in the study of biochemical reaction kinetics and molecular motors.

## Photobleaching FRET

FRET efficiencies can also be inferred from the photobleaching rates of the donor in the presence and absence of an acceptor. This method can be performed on most fluorescence microscopes; one simply shines the excitation light (of a frequency that will excite the donor but not the acceptor significantly) on specimens with and without the acceptor fluorophore and monitors the donor fluorescence (typically separated from acceptor fluorescence using a bandpass filter) over time. The timescale is that of photobleaching, which is seconds to minutes, with fluorescence in each curve being given by

$$(\text{background}) + (\text{constant}) * e^{-(\text{time})/\tau_{pb}}$$

where  $\tau_{pb}$  is the photobleaching decay time constant and depends on whether the acceptor is present or not. Since photobleaching consists in the permanent inactivation of excited fluorophores, resonance energy transfer from an excited donor to an acceptor fluorophore prevents the photobleaching of that donor fluorophore, and thus high FRET efficiency leads to a longer photobleaching decay time constant:

$$E = 1 - \tau_{pb}/\tau'_{pb}$$

where  $\tau'_{pb}$  and  $\tau_{pb}$  are the photobleaching decay time constants of the donor in the presence and in the absence of the acceptor, respectively. (Notice that the fraction is the reciprocal of that used for lifetime measurements).

This technique was introduced by Jovin in 1989. Its use of an entire curve of points to extract the time constants can give it accuracy advantages over the other methods. Also, the fact that time measurements are over seconds rather than nanoseconds makes it easier than fluorescence lifetime measurements, and because photobleaching decay rates do not generally depend on donor concentration (unless acceptor saturation is an issue), the careful control of concentrations needed for intensity measurements is not needed. It is, however, important to keep the illumination the same for the with- and without-acceptor measurements, as photobleaching increases markedly with more intense incident light.

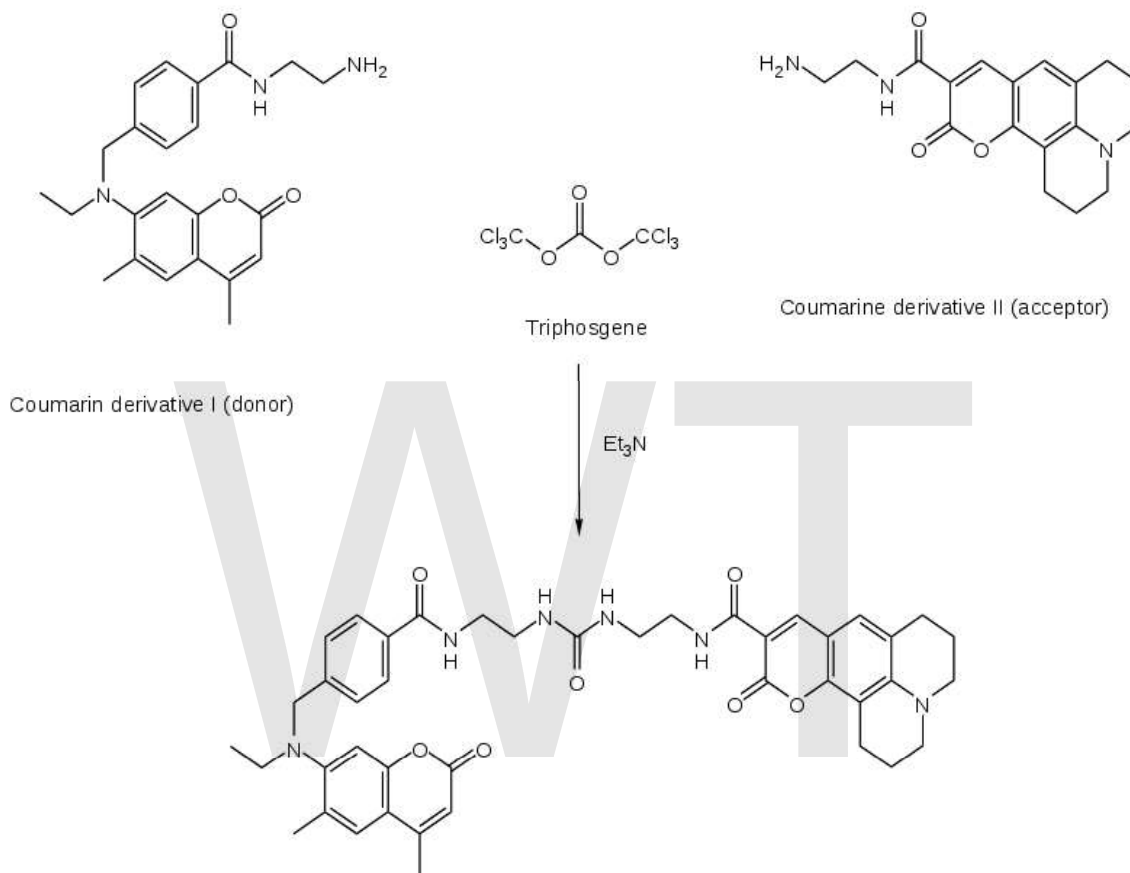
## Other methods

A different, but related, mechanism is Dexter Electron Transfer.

An alternative method to detecting protein-protein proximity is the bimolecular fluorescence complementation (BiFC) where two halves of a YFP are fused to a protein. When these two halves meet they form a fluorophore after about 60 s - 1 hr.

## Application

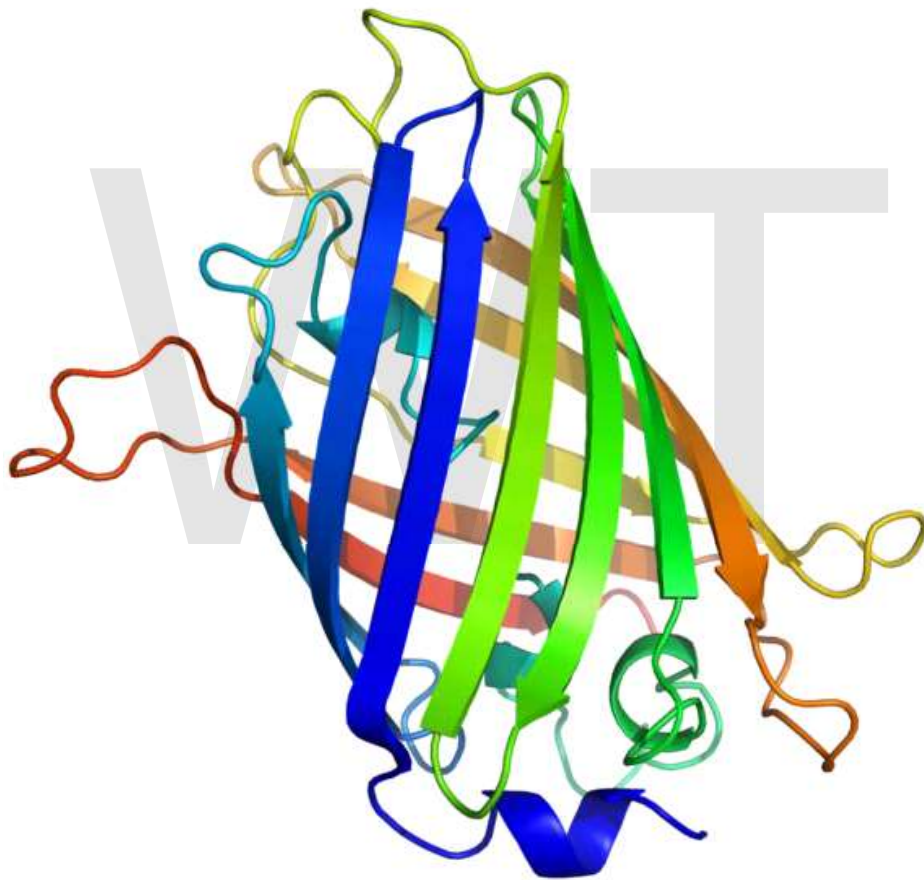
FRET has been applied in an experimental method for the detection of phosgene. In it, phosgene or rather triphosgene as a safe substitute serves as a linker between an acceptor and a donor coumarin (forming urea groups). The presence of phosgene is detected at  $5 \times 10^{-5} \text{M}$  with a typical FRET emission at 464 nm.



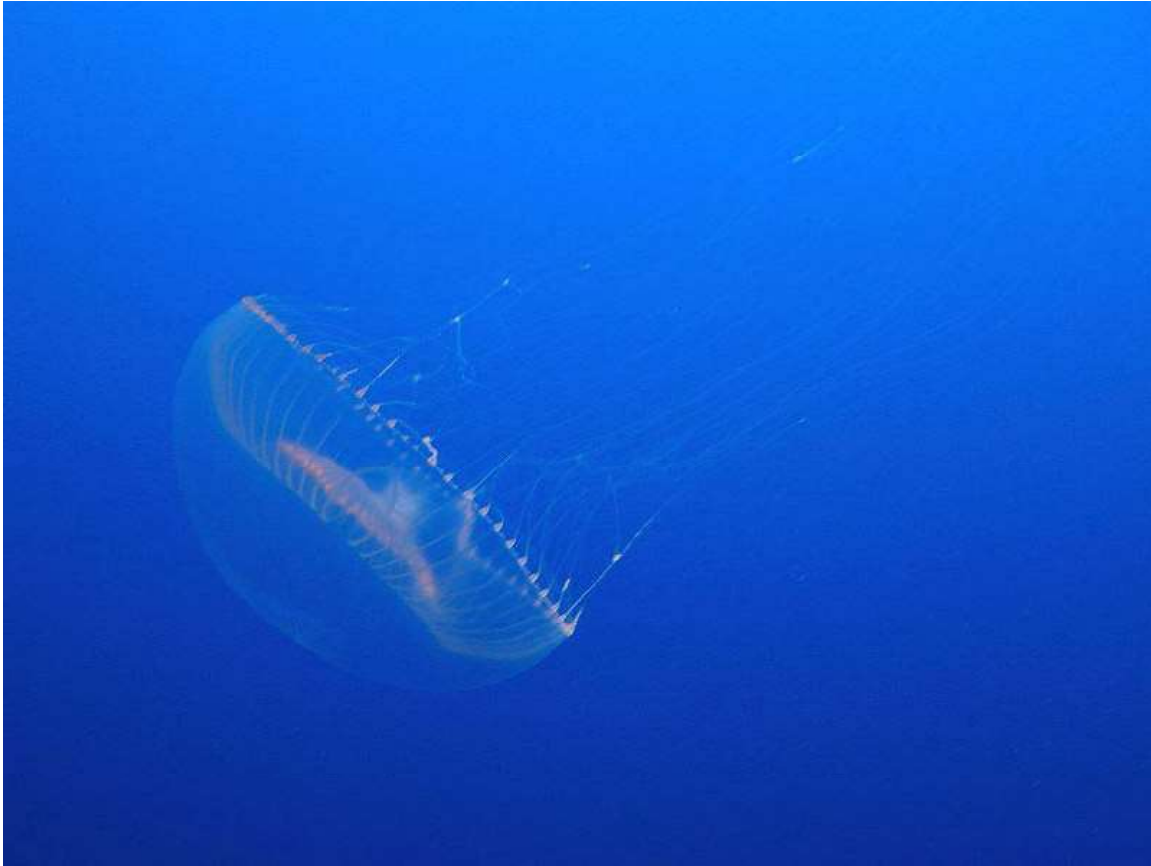
FRET is also used to study lipid rafts in cell membranes.

## Chapter 16

# Green Fluorescent Protein



GFP ribbon diagram. From PDB 1EMA.



*Aequorea victoria*

The **green fluorescent protein (GFP)** is a protein composed of 238 amino acid residues (26.9kDa) that exhibits bright green fluorescence when exposed to blue light. Although many other marine organisms have similar green fluorescent proteins, GFP traditionally refers to the protein first isolated from the jellyfish *Aequorea victoria*. The GFP from *A. victoria* has a major excitation peak at a wavelength of 395 nm and a minor one at 475 nm. Its emission peak is at 509 nm, which is in the lower green portion of the visible spectrum. The GFP from the sea pansy (*Renilla reniformis*) has a single major excitation peak at 498 nm. In cell and molecular biology, the GFP gene is frequently used as a reporter of expression. In modified forms it has been used to make biosensors, and many animals have been created that express GFP as a proof-of-concept that a gene can be expressed throughout a given organism. The GFP gene can be introduced into organisms and maintained in their genome through breeding, injection with a viral vector, or cell transformation. To date, the GFP gene has been introduced and expressed in many bacteria, yeast and other fungi, fish (such as zebrafish), plant, fly, and mammalian cells, including human. Martin Chalfie, Osamu Shimomura, and Roger Y. Tsien were awarded the 2008 Nobel Prize in Chemistry on 10 October 2008 for their discovery and development of the green fluorescent protein.

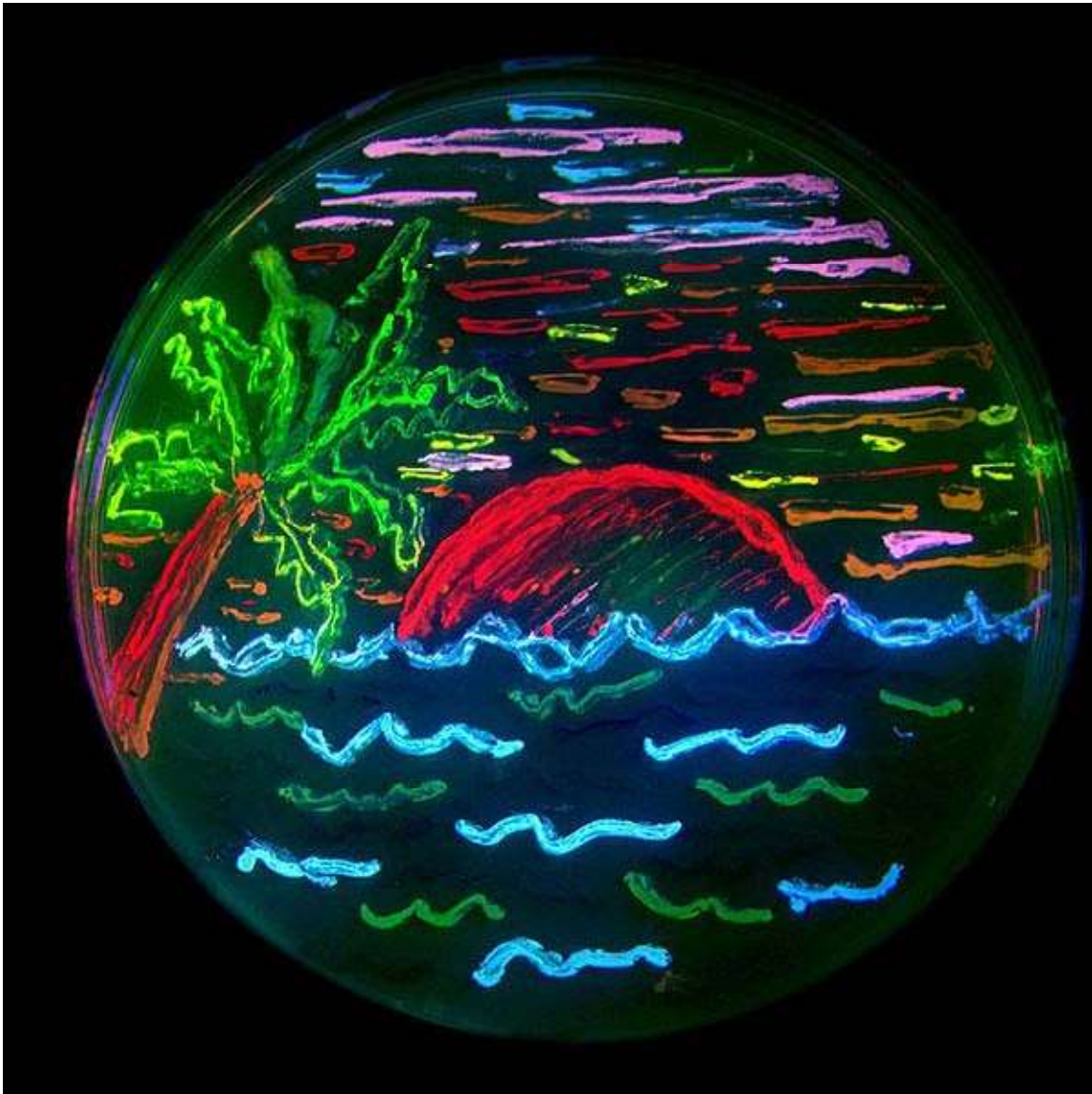
## **History**

### **Wild-type GFP (wtGFP)**

In the 1960s and 1970s, GFP, along with the separate luminescent protein aequorin, was first purified from *Aequorea victoria* and its properties studied by Osamu Shimomura. In *A. victoria*, GFP fluorescence occurs when aequorin interacts with  $\text{Ca}^{2+}$  ions, inducing a blue glow. Some of this luminescent energy is transferred to the GFP, shifting the overall color towards green. However, its utility as a tool for molecular biologists did not begin to be realized until 1992 when Douglas Prasher reported the cloning and nucleotide sequence of wtGFP in *Gene*. The funding for this project had run out, so Prasher sent cDNA samples to several labs. The lab of Martin Chalfie expressed the coding sequence of wtGFP, with the first few amino acids deleted, in heterologous cells of *E. coli* and *C. elegans*, publishing the results in *Science* in 1994. Frederick Tsuji's lab independently reported the expression of the recombinant protein one month later. Remarkably, the GFP molecule folded and was fluorescent at room temperature, without the need for exogenous cofactors specific to the jellyfish. Although this near-wtGFP was fluorescent, it had several drawbacks, including dual peaked excitation spectra, pH sensitivity, chloride sensitivity, poor fluorescence quantum yield, poor photostability and poor folding at 37°C.

The first reported crystal structure of a GFP was that of the S65T mutant by the Remington group in *Science* in 1996. One month later, the Phillips group independently reported the wild-type GFP structure in *Nature Biotech*. These crystal structures provided vital background on chromophore formation and neighboring residue interactions. Researchers have modified these residues by directed and random mutagenesis to produce the wide variety of GFP derivatives in use today. Martin Chalfie, Osamu Shimomura and Roger Y. Tsien share the 2008 Nobel Prize in Chemistry for their discovery and development of the green fluorescent protein.

## GFP derivatives



The diversity of genetic mutations is illustrated by this San Diego beach scene drawn with living bacteria expressing 8 different colors of fluorescent proteins.

Due to the potential for widespread usage and the evolving needs of researchers, many different mutants of GFP have been engineered. The first major improvement was a single point mutation (S65T) reported in 1995 in *Nature* by Roger Tsien. This mutation dramatically improved the spectral characteristics of GFP, resulting in increased fluorescence, photostability, and a shift of the major excitation peak to 488 nm, with the peak emission kept at 509 nm. This matched the spectral characteristics of commonly available FITC filter sets, increasing the practicality of use by the general researcher. A 37 °C folding efficiency (F64L) point mutant to this scaffold yielding enhanced GFP (EGFP) was discovered in 1995 by the lab of Ole Thastrup. EGFP allowed the practical use of GFPs in mammalian cells. EGFP has an extinction coefficient (denoted  $\epsilon$ ) of

$55,000 \text{ M}^{-1}\text{cm}^{-1}$ . The fluorescence quantum yield (QY) of EGFP is 0.60. The relative brightness, expressed as  $\epsilon \cdot \text{QY}$ , is  $33,000 \text{ M}^{-1}\text{cm}^{-1}$ . Superfolder GFP, a series of mutations that allow GFP to rapidly fold and mature even when fused to poorly folding peptides, was reported in 2006.

Many other mutations have been made, including color mutants; in particular, blue fluorescent protein (EBFP, EBFP2, Azurite, mKalama1), cyan fluorescent protein (ECFP, Cerulean, CyPet), and yellow fluorescent protein derivatives (YFP, Citrine, Venus, YPet). BFP derivatives (except mKalama1) contain the Y66H substitution. The critical mutation in cyan derivatives is the Y66W substitution, which causes the chromophore to form with an indole rather than phenol component. Several additional compensatory mutations in the surrounding barrel are required to restore brightness to this modified chromophore due to the increased bulk of the indole group. The red-shifted wavelength of the YFP derivatives is accomplished by the T203Y mutation and is due to  $\pi$ -electron stacking interactions between the substituted tyrosine residue and the chromophore. These two classes of spectral variants are often employed for fluorescence resonance energy transfer (FRET) experiments. Genetically-encoded FRET reporters sensitive to cell signaling molecules, such as calcium or glutamate, protein phosphorylation state, protein complementation, receptor dimerization, and other processes provide highly specific optical readouts of cell activity in real time.

Semirational mutagenesis of a number of residues led to pH-sensitive mutants known as pHluorins, and later super-ecliptic pHluorins. By exploiting the rapid change in pH upon synaptic vesicle fusion, pHluorins tagged to synaptobrevin have been used to visualize synaptic activity in neurons.

Redox sensitive versions of GFP (roGFP) were engineered by introduction of cysteines into the beta barrel structure. The redox state of the cysteines determines the fluorescent properties of roGFP.

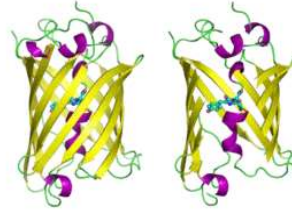
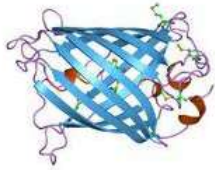
The nomenclature of modified GFPs is often confusing due to overlapping mapping of several GFP versions onto a single name. For example, **mGFP** often refers to a GFP with an N-terminal palmitoylation that causes the GFP to bind to cell membranes. However, the same term is also used to refer to monomeric GFP, which is often achieved by the dimer interface breaking A206K mutation. Wild-type GFP has a weak dimerization tendency at concentrations above 5 mg/mL. mGFP also stands for "modified GFP," which has been optimized through amino acid exchange for stable expression in plant cells.

## **Structure**

GFP has a typical beta barrel structure, consisting of one  $\beta$ -sheet with alpha helix(s) containing the chromophore running through the center. Inward-facing sidechains of the barrel induce specific cyclization reactions in the tripeptide Ser65–Tyr66–Gly67 that lead to chromophore formation. This process of post-translational modification is referred to as *maturation*. The hydrogen-bonding network and electron-stacking interactions with

these sidechains influence the color of wtGFP and its numerous derivatives. The tightly packed nature of the barrel excludes solvent molecules, protecting the chromophore fluorescence from quenching by water.

### Green fluorescent protein

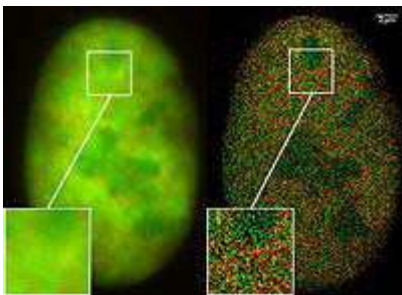


Structure of the Aequorea victoria green fluorescent protein.

GFP molecules drawn in cartoon style, one fully and one with the side of the beta barrel cut away to reveal the chromophore (highlighted as ball-and-stick). From PDB 1GFL.

Identifiers	
Symbol	GFP
Pfam	PF01353
Pfam clan	CL0069
InterPro	IPR011584
SCOP	1ema
<b>Available protein structures:</b>	

### Use



Superresolution with two fusion proteins (GFP-Snf2H and RFP-H2A), Co-localisation studies (2CLM) in the nucleus of a bone cancer cell. 120.000 localized molecules in a widefield area(470  $\mu\text{m}^2$ )

The availability of GFP and its derivatives has thoroughly redefined fluorescence microscopy and the way it is used in cell biology and other biological disciplines. While most small fluorescent molecules such as FITC (fluorescein isothiocyanate) are strongly

phototoxic when used in live cells, fluorescent proteins such as GFP are usually much less harmful when illuminated in living cells. This has triggered the development of highly automated live-cell fluorescence microscopy systems, which can be used to observe cells over time expressing one or more proteins tagged with fluorescent proteins. For example, GFP had been widely used in labelling the spermatozoa of various organisms for identification purposes as in *Drosophila melanogaster*, where expression of GFP can be used as a marker for a particular characteristic. GFP can also be expressed in different structures enabling morphological distinction. In such cases, the gene for the production of GFP is spliced into the genome of the organism in the region of the DNA that codes for the target proteins and that is controlled by the same regulatory sequence; that is, the gene's regulatory sequence now controls the production of GFP, in addition to the tagged protein(s). In cells where the gene is expressed, and the tagged proteins are produced, GFP is produced at the same time. Thus, only those cells in which the tagged gene is expressed, or the target proteins are produced, will fluoresce when observed under fluorescence microscopy. Analysis of such time lapse movies has redefined the understanding of many biological processes including protein folding, protein transport, and RNA dynamics, which in the past had been studied using fixed (i.e., dead) material.

The Vertico SMI microscope using the SPDM Phymod technology uses the so-called "reversible photobleaching" effect of fluorescent dyes like GFP and its derivatives to localize them as single molecules in an optical resolution of 10 nm. This can also be performed as a co-localization of two GFP derivatives (2CLM).

Another powerful use of GFP is to express the protein in small sets of specific cells. This allows researchers to optically detect specific types of cells *in vitro* (in a dish), or even *in vivo* (in the living organism). Genetically combining several spectral variants of GFP is a useful trick for the analysis of brain circuitry (Brainbow). Other interesting uses of fluorescent proteins in the literature include using FPs as sensors of neuron membrane potential, tracking of AMPA receptors on cell membranes, viral entry and the infection of individual influenza viruses and lentiviral viruses, etc.

It has also been found that new lines of transgenic GFP rats can be relevant for gene therapy as well as regenerative medicine. By using "high-expresser" GFP, transgenic rats display high expression in most tissues, and many cells that have not been characterized or have been only poorly characterized in previous GFP-transgenic rats. Through its ability to form internal chromophore without requiring accessory cofactors, enzymes or substrates other than molecular oxygen, GFP makes for an excellent tool in all forms of biology.

### ***GFP in nature***

The purpose of both bioluminescence and GFP fluorescence in jellyfish is unknown. GFP is co-expressed with aequorin in small granules around the rim of the jellyfish bell. The secondary excitation peak (480 nm) of GFP does absorb some of the blue emission of aequorin, giving the bioluminescence a more green hue. The serine 65 residue of the GFP chromophore is responsible for the dual-peaked excitation spectra of wild-type GFP. It is

conserved in all three GFP isoforms originally cloned by Prasher. Nearly all mutations of this residue consolidate the excitation spectra to a single peak at either 395 nm or 480 nm. The precise mechanism of this sensitivity is complex, but, it seems, involves donation of a hydrogen from serine 65 to glutamate 222, which influences chromophore ionization. Since a single mutation can dramatically enhance the 480 nm excitation peak, making GFP a much more efficient partner of aequorin, *A. victoria* appears to evolutionarily prefer the less-efficient, dual-peaked excitation spectrum. Roger Tsien has speculated that varying hydrostatic pressure with depth may effect serine 65's ability to donate a hydrogen to the chromophore and shift the ratio of the two excitation peaks. Thus, the jellyfish may change the color of its bioluminescence with depth. However, a collapse in the population of jellyfish in Friday Harbor, where GFP was originally discovered, has hampered further study of the role of GFP in the jellyfish's natural environment.

### ***GFP in fine art***



Julian Voss-Andreae's GFP-based sculpture *Steel Jellyfish* (2006). The image shows the stainless-steel sculpture on display at Friday Harbor Laboratories on San Juan Island (Wash., USA), the place of GFP's discovery.

Julian Voss-Andreae, a German-born artist specializing in "protein sculptures," created sculptures based on the structure of GFP, including the 5'6" (1.70 m) tall "Green Fluorescent Protein" (2004) and the 4'7" (1.40 m) tall "Steel Jellyfish" (2006). The latter sculpture is currently located at the place of GFP's discovery by Shimomura in 1962, the University of Washington's Friday Harbor Laboratories.

Eduardo Kac has also done some work with GFP, the most notable one being the "GFP Bunny," Alba. Kac commissioned a French laboratory to create a green-fluorescent rabbit, which is the subject of a series of his art pieces.

### ***Transgenic pets***

Alba, a fluorescent rabbit, was commissioned by Eduardo Kac using GFP for purposes of art and social commentary. The US company Yorktown Technologies markets to aquarium shops green fluorescent zebrafish (GloFish) that were initially developed to detect pollution in waterways. NeonPets, a US based company markets green fluorescent mice to the pet industry as NeonMice. Green fluorescent pigs, known as Noels were bred by a group of researchers led by Wu Shinn-Chih at the Department of Animal Science and Technology at National Taiwan University.

WWT