Imaging dynamic processes

Introduction

In the previous lesson, we have introduced methods to improve classical fluorescence microscopy in its image resolution and signal-to-noise ratio. However, these methods have in common that only "snapshots" of the cell at a certain time are taken. We are only able to see one exact state of the cell, without being able to set it into context with previous or subsequent events. But cells are not static: Cells are very adaptive to their environment and therefore highly dynamic! The molecules in a cell are in constant movement. Macromolecular structures have to be assembled, disassembled, and reorganized during a cell's life cycle. Parts of molecular machinery move around in the cell and interact with each other. By watching the movie below, you can get a sense of these intracellular dynamics.

`https://www.youtube.com/watch?v=DR80Huxp4y8`

The movement of cellular macromolecules can be mainly divided into two categories: The first one is diffusion, which refers to the Brownian or thermal motion of molecules. The second one is active transport, which is driven by the hydrolysis of energy-rich molecules such as ATP. By active transport, cells are able to speed up the movement and restrict the direction of molecules. This is especially needed in eukaryotic cells, which are too large to rely on diffusion alone for transportation. Usually, cells use motor proteins to mediate the transport of cargo molecules along the cytoskeleton. Additionally, molecular movement is also influenced by the high concentration of biomolecules in the cell. Cells are incredibly crowded! This is illustrated in figure 4-1 by D. Goodsell, who drew the interior of a eukaryotic cell based on the measured concentrations and the dimensions of individual cellular components.

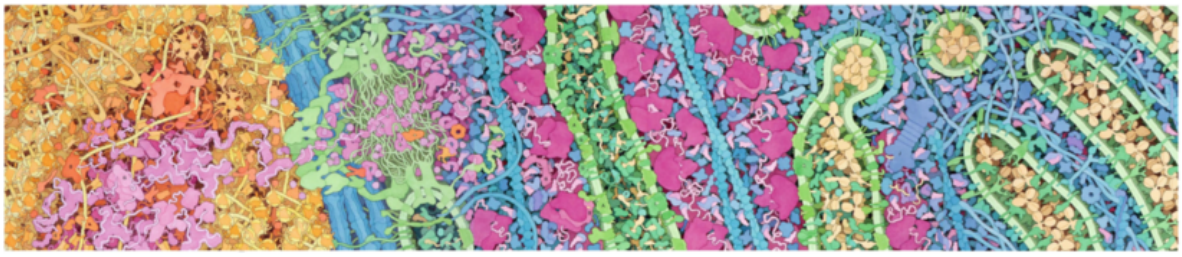

Figure 4-1 Illustration of a section of a eukaryotic cell encompassing part of the nucleus and the cytoplasm by David Goodsell. The nuclear interior is shown on the left, bounded by the nuclear envelope with a single nuclear pore complex shown. The cytoplasm is on the right. Because of this tight packaging, molecules regularly bump into each other and cannot diffuse freely. (Adapted from *The Machinery of Life*, D. Goodsell, Springer)

Fluorescent proteins can be used to visualize dynamic processes

How can we measure the actual motion of biomolecules in the cell? Here, the fluorescent property of certain molecules comes into play. Fluorescent molecules can be used to specifically tag proteins and make their location visible by excitation with specific wavelengths in fluorescence microscopy. Most organic fluorescent molecules are chemically synthesized and then introduced into the cell. Others, such as green fluorescent protein (GFP), are encoded by a single gene, which can be introduced into the cell's genome. GFP is often used as a reporter molecule. When the GFP gene is placed under the same transcriptional control as a gene of interest, a fusion protein between GFP and the protein of interest is made. This fusion protein can still fold and function normally, and the GFP fluorescence allows the observation of the protein in a fluorescence microscope (see figure 4-2).

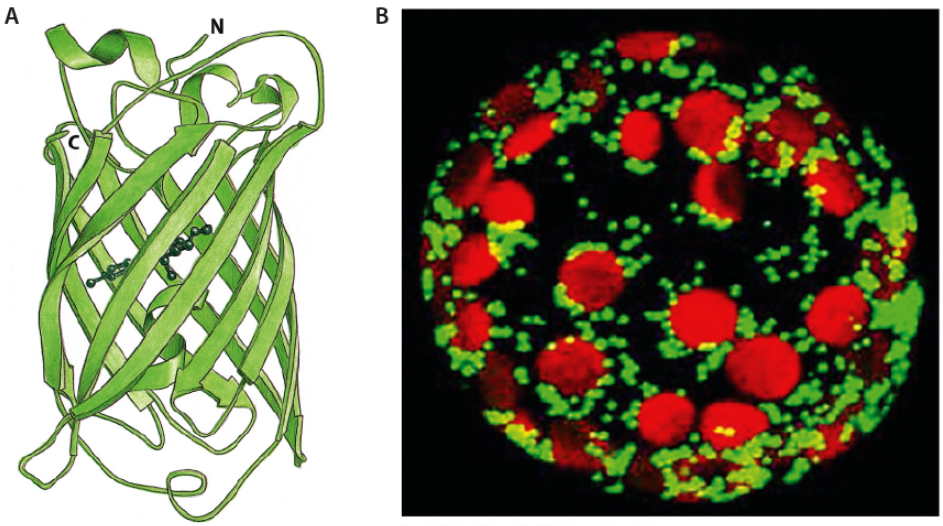

Figure 4-2 Green fluorescent protein (GFP) and GFP-tagged mitochondrial proteins. **(A)** The structure of GFP, shown here schematically, highlights the eleven β strands that form the staves of a barrel. Buried within the barrel is the active chromophore (dark green) that is formed post-translationally from the protruding side chains of three amino-acid residues. **(B)** The living cell from a tobacco plant is expressing high levels of GFP fused to a protein that is targeted to mitochondria, which accordingly appear green. The mitochondria are seen to cluster around the chloroplasts, whose chlorophyll autofluorescence marks them out in red. (Adapted from Figure 9-22 and 9-24, *Molecular Biology of the Cell*, Alberts *et al.*, 6th edition, Garland Science)

However, as stated above, proteins are rarely static. Thus, instead of taking static images of fluorescent molecules, how can we visualize their motion within a cell? The simplest way to show the intracellular dynamics is to take several images at different time points, as shown in figure 4-3. This way, we can clearly see if and how molecules are moving around. We can also observe if they move randomly, by diffusion, or if it is a directed movement. But what we cannot measure by this method is the underlying kinetics of the movement.

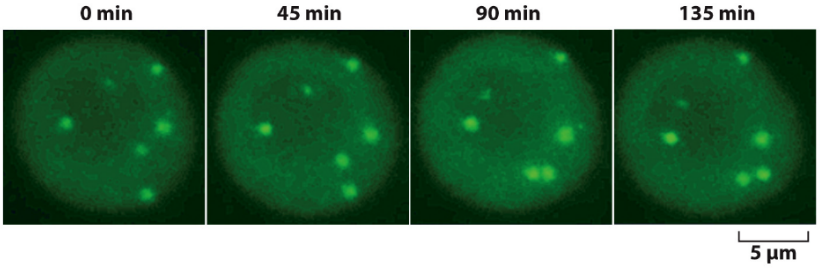

Figure 4-3 Dynamics of GFP tagging. This sequence of micrographs shows a set of three-dimensional images of a living nucleus taken over the course of 135 min. Tobacco cells have been stably transformed with GFP fused to a spliceosomal protein that is concentrated in small nuclear bodies. These fluorescent bodies, easily visible in a living cell with confocal microscopy, are dynamic structures that move around within the nucleus. (Adapted from Figure 9-25, *Molecular Biology of the Cell*, Alberts *et al.*, 6th edition, Garland Science)

Diffusion can be measured using photobleaching

If we want to quantify the motion of biomolecules in cells, we can use a method called photobleaching. This is a chemical modification of the fluorophore molecule that is induced by exposure to light of high

intensity, resulting in a loss of fluorescence. The most common method which uses photobleaching is called **fluorescence recovery after photobleaching (FRAP)**. The principle is quite simple: A strong laser beam is focused to photobleach fluorescent molecules in a certain part of the cell. We can then observe the recovery of the fluorescence, which is due to unbleached fluorophores from other parts of the cell that diffuse into the bleached area. This happens normally over the course of seconds or minutes. If the observed protein is highly mobile, recovery will be very fast; if the protein is immobile (e.g., because it is attached to the plasma membrane or its location is restricted to a certain organelle), no recovery will occur. If we observe that the fluorescence does not reach its initial level, this indicates that a fraction of proteins is immobile and cannot diffuse into the bleached area. By plotting the mean fluorescence in the bleached area versus the time after photobleaching, kinetic coefficients can be calculated (for example, the diffusion coefficient of a protein or active-transport rates, see figure 4-4). But keep in mind that the photobleaching process does not influence the protein concentration in the bleached area. It just makes the molecules invisible to measurement, because the fluorophore has been inactivated. The kinetics of the recovery therefore represent the normal dynamic behavior of the observed protein.

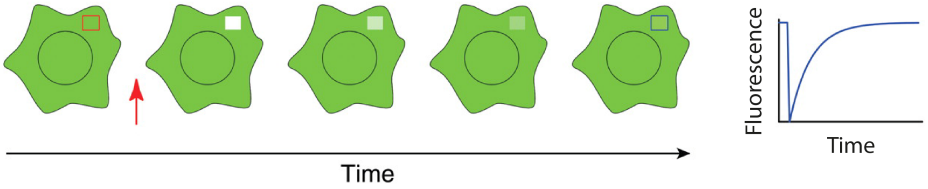

Figure 4-4 FRAP technique. Schematic illustration of a typical cell with nucleus and cytoplasm. Here, a protein that is ubiquitous within the cell is tagged with GFP. The photoperturbed region is marked by a red boundary. The arrow indicates the photoperturbation event. By observing the fluorescence recovery and plotting it as a function of the time, one can deduce, e.g., the diffusion coefficient of the protein. (Adapted from A. Bancaud *et al.*, Cold Spring Harb. Protoc., 2010)

Which biological problems can this technique address? Originally, the FRAP method was intended to observe the motility of an individual lipid molecule in the cell membrane. But applications were soon extended. In the example shown in figure 4-5, the enzyme galactosyltransferase, which travels between the endoplasmic reticulum and the Golgi, was tagged with GFP. The Golgi of one of two observed cells was then selectively photobleached. The fluorescence recovery results from new fluorescent molecules traveling from the ER to the Golgi.

Not only diffusion, but also protein binding and dissociation rates can be measured using FRAP. If the fluorescently tagged protein binds to static receptors in the bleached area, the recovery of the fluorescence reveals information of the association and dissociation constants. If the protein is tightly bound and disassociates slowly, the recovery needs more time. On the other hand, if the recycling of the bound protein is rapid, there is a much faster recovery.

Monitoring protein-protein interactions by FRET

With FRAP, we can study the motion of molecules. But apart from knowing the location and movement of a protein within the cell, we would also like to know whether and how the protein interacts with other molecules. Dynamic protein interactions play a crucial role in many cellular regulation mechanisms, such as signal-transduction pathways and transcriptional regulation. As we have seen in previous lessons, the image resolution of fluorescence microscopy is limited by the diffraction of light to approximately 200nm. Since interacting molecules need to approach each other to a distance of less than a few nanometers, visualizing such interactions by light microscopy seems impossible.

However, using **Fluorescence resonance energy transfer** or **Förster resonance energy transfer** (both abbreviated **FRET**) we can actually observe molecular interactions. Using this technique, we can detect that two molecules are within several nanometers proximity.

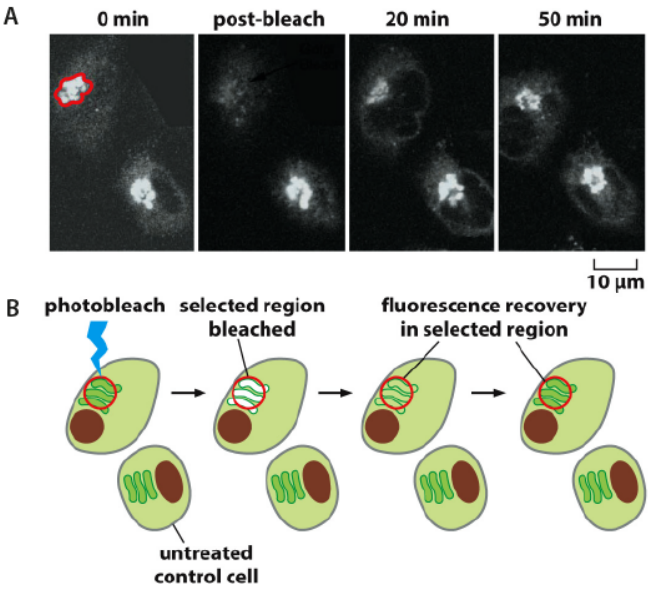

Figure 4-5 Fluorescence recovery after photobleaching (FRAP). A strong focused pulse of laser light will extinguish, or bleach, the fluorescence of GFP. By selectively photobleaching a set of fluorescently tagged protein molecules within a defined region of a cell, the microscopist can monitor recovery over time, as the remaining fluorescent molecules move into the bleached region. **(A)** The experiment shown uses monkey cells in culture that express galactosyltransferase, an enzyme that constantly recycles between the Golgi and the ER. The Golgi in one of the two cells is selectively photobleached, while the production of new fluorescent protein is blocked by treating the cells with cycloheximide. The recovery, resulting from fluorescent enzyme molecules moving from the ER to the Golgi, can then be followed over a period of time. **(B)** Schematic diagram of the experiment shown in A. (Adapted from Figure 9-29, *Molecular Biology of the Cell*, Alberts *et al.*, 6th edition, Garland Science)

But how does it work? In the lecture, we have seen that there is an energy shift between excitation and emission of a fluorophore. This shift is the key to how FRET works: The two molecules whose interaction we want to study are both labeled with different fluorophores. The fluorophores are chosen such that the emission spectrum of one (the donor, blue in figure 4-6A-C) overlaps with the excitation spectrum of the other one (the acceptor, green in figure 4-6A-C).

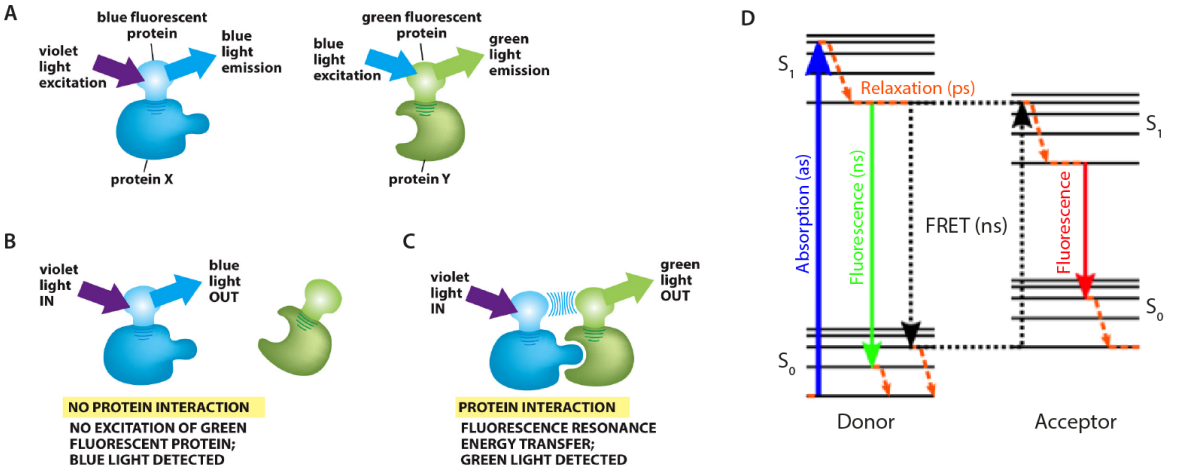

Figure 4-6 Fluorescence resonance energy transfer (FRET). (A) In this example, protein X is coupled to a blue fluorescent protein, which is excited by violet light (370–440 nm) and emits blue light (440–480 nm); protein Y is coupled to a green fluorescent protein, which is excited by blue light (440–480 nm) and emits green light (510 nm). **(B)** If protein X and Y do not interact, illuminating the sample with violet light yields fluorescence from the blue fluorescent protein only. **(C)** When protein X and protein Y interact, the resonance transfer of energy, FRET, can now occur. Illuminating the sample with violet light excites the blue fluorescent protein, which transfers its energy to the green fluorescent protein, resulting in an emission of green light. **(D)** Jablonski energy diagram of a FRET. (A-C: Adapted from Figure 9-26, *Molecular Biology of the Cell*, Alberts *et al.*, 6th edition, Garland Science)

In the experiment, only the excitation wavelength of the donor is used for illumination (violet excitation). If the two molecules are far apart, we will observe light at the emission wavelength of the donor (blue light). However, if the two molecules bind, and therefore come into close proximity, the donor fluorophore can transfer the energy from the absorbed light directly to the acceptor fluorophore. In this case, we will not observe emission from the donor, but only from the acceptor (green light). The transfer of energy between the two fluorophores happens by resonance and is therefore non-radiative, i.e., no energy is lost in the transfer (see figure 4-6D).

The FRET efficiency

There are several criteria that must be fulfilled for FRET to happen. In addition to the overlapping excitation and emission spectra, the two fluorophores must be in close proximity to each other (usually 1-10 nm). Furthermore, the lifetime of the donor molecule must be long enough to allow the energy transfer to occur. The efficiency of the FRET process (E_{FRET}) depends on the inversed sixth power of the distance between the donor and the acceptor (see figure 4-7). The transfer efficiency is a value between 0 and 1, where 1 means that the entire energy used to excite the donor is transferred to excite the acceptor. This is the case if donor and acceptor are in close proximity. If the two molecules are farther than 10nm apart, the transfer efficiency decreases rapidly.

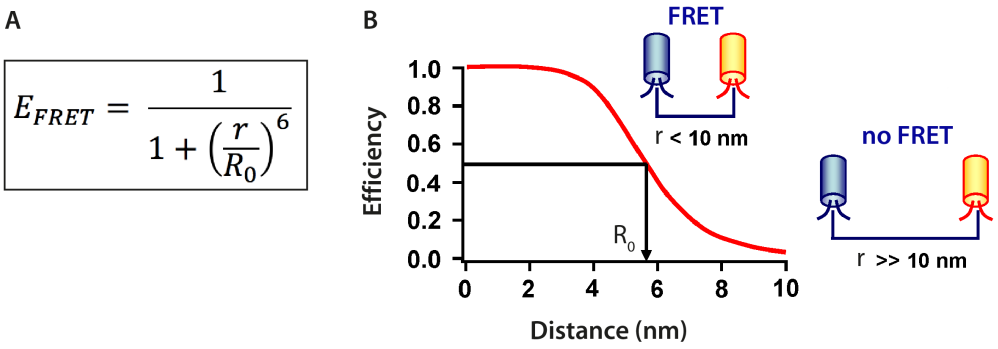

Figure 4-7 Formula and diagram of FRET efficiency as a function of the donor-to-acceptor distance. (A) Formula describing the transfer efficiency, where *r* is the donor-to-acceptor distance and R_0 is the so-called *Förster distance*, describing the distance of a FRET pair where transfer efficiency is 50%. The Förster distance varies for each donor-acceptor pair and lies for common fluorophore pairs between 2 and 8 nm. **(B)** In close proximity of donor and acceptor, the efficiency approaches 1; at a distance greater than 10 nm, the efficiency drops towards 0. This is an example of one fluorophore pair; the exact function course is different for each pair. (Adapted from Wikipedia: *Förster-Resonanzenergietransfer*)

Example of an application of FRET

The FRET efficiency can be quantified in a number of different ways, for example, by measuring the reduction of the donor fluorescence in the presence of the acceptor. The strong distance-dependence of the FRET efficiency is widely used to study the structure and interactions of proteins. The distance over which energy can be transferred strongly depends on the fluorophore pair used in the study. An ideal pair of fluorophores has a strong overlap of the donor emission and the acceptor excitation spectra. In general, a higher overlap between the spectra yields larger Förster distance values, which means that energy transfer can already happen at a greater distance between the two fluorophores. You can see an example of such a fluorophore pair in figure 4-8. Here, Karsten Weis and his colleagues used the FRET pair CFP (cyan fluorescent protein) and YFP (yellow fluorescent protein) to measure the distribution of Ran-GTP and Ran-GDP in mitotic somatic cells. You can see that Ran-GTP binds

to a complex consisting of a Ran-binding domain and two fluorophores. When Ran-GTP is not bound to the complex, the two fluorophores are in close proximity, allowing energy transfer to happen. When the complex is illuminated by a laser beam, light emitted from exciting YFP (525nm) can be detected. However, if Ran-GTP is bound to the complex, this changes the conformation of the complex, leading to a separation of the two fluorophores. FRET does not occur anymore, as detected by a change of the emission wavelength to the CFP emission peak.

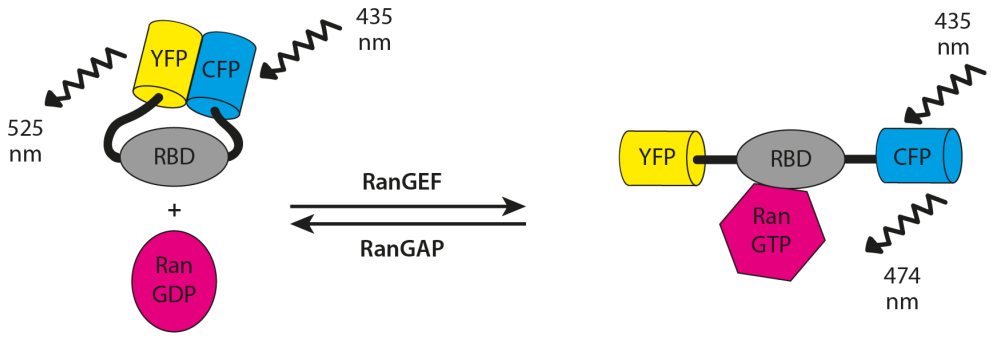

Figure 4-8 Schematic illustration of a FRET probe used to determine the concentration of Ran-GTP in different parts of the cell. The probe consists of YFP and CFP fused to a central Ran-binding domain (RBD). If Ran-GTP binds to the probe, YFP and CFP are no longer in close proximity and no FRET occurs. However, if the dephosphorylated version (Ran-GDP), to which the RBD has low affinity, is more abundant, YFP and CFP are close together, resulting in FRET. In this case, light emitted by YFP at 525 nm can be detected. Ran-GEF and Ran-Gap catalyze the conversion between Ran-GTP and Ran-GDP. (Adapted from P. Kaláb *et al.*, Science, 2002)

To quantify the interactions between the protein complex and Ran-GTP, the CFP-emission and the YFP-emission (resulting from FRET) were compared. By measuring this ratio in different parts of a cell, the distribution of Ran-GTP within the cell could be traced. This example shows that FRET cannot only be used to study certain protein-protein interactions, but can also monitor the concentration of a molecule in a cell. Thus, the FRET technique has a wide range of applications. It has been used for measuring structures, conformational changes, interactions between molecules, and biochemical events.

Fluorescence live imaging is an integral part of modern cell biology

In this lesson, we introduced two new techniques to measure dynamic events in cells. In FRAP, the phenomenon of photobleaching is used to observe the motion of molecules. It can provide information about the kinetic parameters of a protein, such as diffusion coefficients, active transport rates, or binding and dissociation rates from other proteins. In FRET, the proximity between two fluorophores leads to a transfer of energy and therefore to a change of the observed emission wavelength. Among many other applications, it can be used to monitor protein interactions and conformational changes of proteins. The examples discussed here show that the properties of fluorescence can be used in various ways to study the dynamics of a cell.