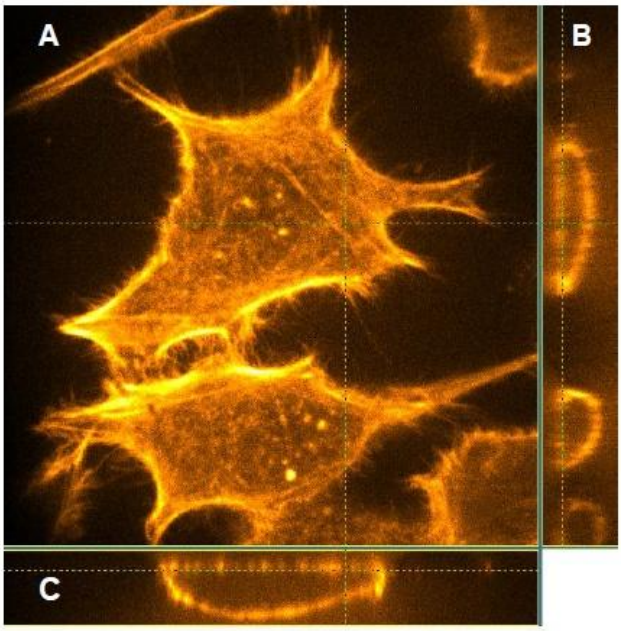
Biophysics Lab Course

ulm university universität ulm

Live Cell Imaging

Fluorescence imaging of the actin cytoskeleton with a spinning disk confocal microscope
Sensing of calcium signals and transport of fusion proteins in living cells

Subjects: Fluorescence: absorption, emission, Jablonski diagram, Stokes shift
Microscopy: optical path in the microscope, diffraction limit.
Immunostaining, fluorescent proteins, calcium sensors.



3D reconstruction of rhodamine phalloidin stained actin cytoskeleton of HeLa cells

Experiments:

Using fluorescence microscopy, cellular proteins can be visualized by a variety of highly sensitive and selective labeling strategies. The spinning disk confocal fluorescence microscope provides the opportunity to acquire fast image stacks with high z-resolution. In the first experiment, the actin cytoskeleton of fixed HeLa cells is stained by rhodamine phalloidin and visualized in a 3D-reconstruction of an image stack. In the second part, intracellular calcium signals are observed in living cells after receptor stimulation. In the third experiment, the photoconvertible fluorescence protein EOS fused to a mitochondrial targeting sequence provides insights into the dynamics of the mitochondrial network.

Introduction

Fluorescence

The processes which result from the absorption of excitation light and lead to the emission of fluorescence are often represented by energy diagrams (Fig 1), which are named after the Polish physicist Alexander Jablonski, the founder of modern fluorescence spectroscopy.

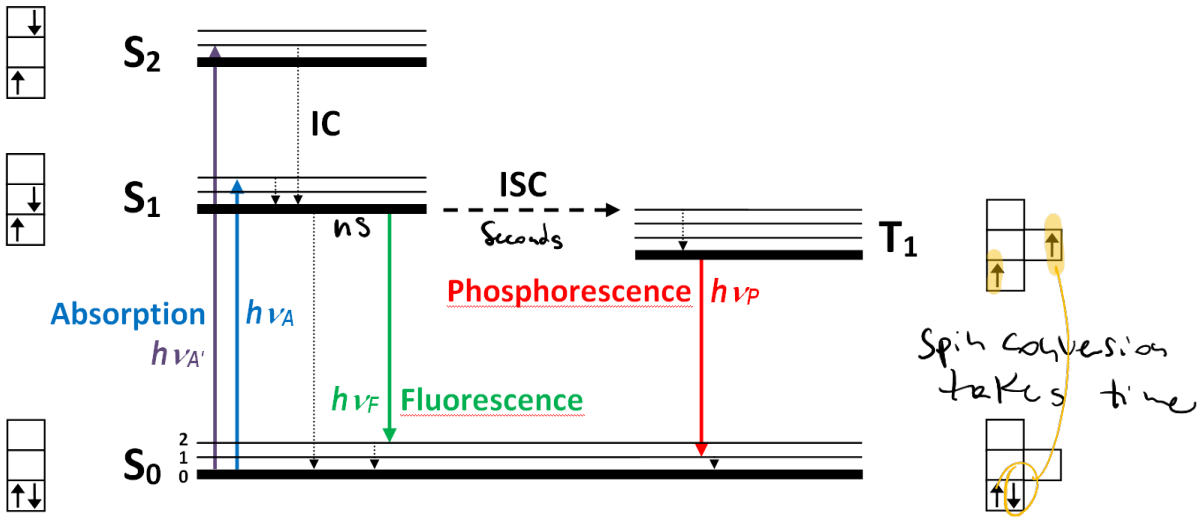


Fig. 1: Jablonski diagram showing possible states and photophysical processes

A Jablonski diagram visualizes the electronic states of a fluorophore and the transitions which may occur between them. Many fluorophores have aromatic ring structures. Such molecules have delocalized electrons in so-called binding π-orbitals. The electrons of these orbitals achieve a higher orbital after light absorption (π*). In binding orbitals electrons are normally present with antiparallel spins ─ an arrangement which characterizes the so-called singlet states (S_0, S_1, S_2). The absorption of a stimulating photon ($h\nu_A$) lifts an electron from the ground state S_0 into one of the excited states S_1 or S_2. This process is extremely fast (on the order of ~ 10^{-15} s). While the electronically excited state S_2 rapidly converts to S_1 without photon emission, the transition from S_1 to the ground state S_0 can either happen radiationless (internal conversion, IC) or by emission of a fluorescence photon ($h\nu_F$). The energy of the emitted photon is always lower than that of the absorbed photon ─ hence, the wavelength of fluorescence is larger, which is called Stokes' shift. The reason for this lies in the relaxation of vibrations, which were excited together with the electronic transitions and are finally converted into heat. The average dwell time in the excited state (fluorescence lifetime) lies in the range of a few nanoseconds for most fluorophores. In some compounds, a transition from an excited singlet state into a triplet state (T_1) can occur. In this process called intersystem

crossing (ISC), a spin flip of the excited electron occurs. The return to the ground state also requires a spin flip, which makes it the transition unlikely. Thus the transition rates are low ($1\text{-}10^6$ per s), which is the reason for the slow decay of phosphorescence which can be seen on toys that glow in the dark.

Fluorescence Microscopy

In conventional fluorescence microscopy a sample is illuminated over a large area, and the resulting fluorescence image is viewed through an emission filter by eye or with a camera (Fig. 2). From the white light from a xenon or mercury arc lamp the wavelength suitable for the excitation of the fluorochrome is selected by an excitation filter.

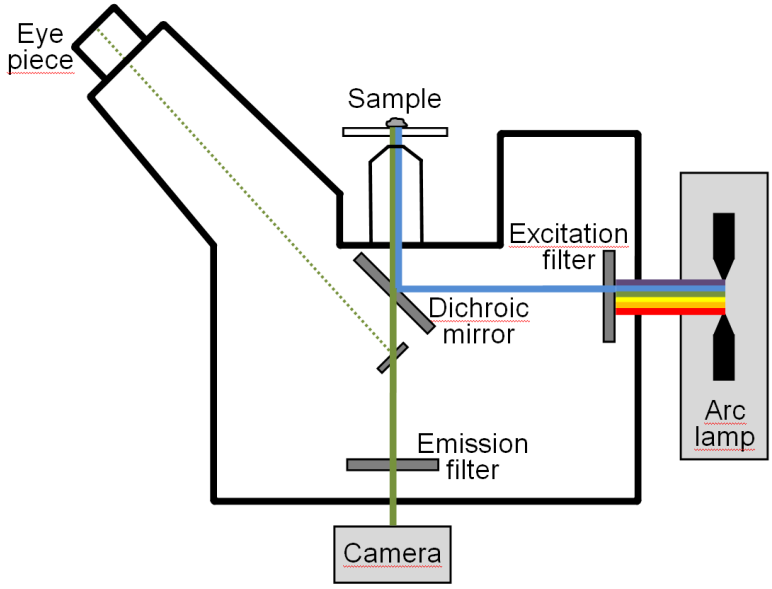


Fig. 2: Schematic of light pathway in a conventional widefield fluorescence microscope

Inside the microscope, the light is reflected by a dichroic mirror onto the sample. Typical dichroic mirrors have a cut-on wavelength: light with shorter wavelengths is reflected while longer wavelengths are transmitted. The mirror is selected such that the critical wavelength lies between the excitation and emission maximum of the fluorochrome. Thus, the excitation light is directed through the lens onto the sample, while the longer-wavelength fluorescence passes from the sample through the dichroic mirror to the eyepiece or to a camera. A high degree of separation of excitation and fluorescence light throughout this optical system is required for attaining good images. Filter sets with suitable combinations of filters and dichroic mirrors can be purchased for each commonly used fluorescent dye.

For the experiments in this course, a confocal spinning disk laser scanning microscope is used (Fig. 3). Confocal imaging provides improved axial resolution over conventional widefield fluorescence microscopy. Therefore it is suitable for 'optical sectioning' by aquiring stacks of images at different z-positions which can finally be combined to a 3D-reconstruction of the sample.

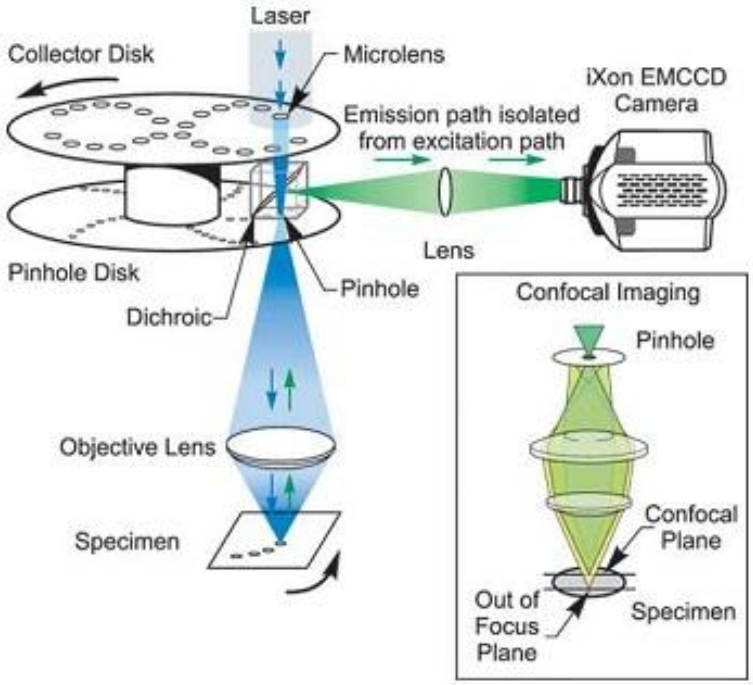


Fig. 3: Schematic setup of a spinning disk confocal fluorescence microscope
(source: andor.oxinst.com/learning/view/article/confocal-dual-spinning-disk)

In contrast to a time consuming single-point scanning, the spinning disk microscope does a simultaneous confocal imaging through ~1000 pinholes at a time and thus enables fast optical sectioning or kinetic image series.

To search for a region of interest in a sample a halogen lamp is used for standard brightfield imaging. When a feasible part of the sample is found, the microscope is switched to confocal laser scanning mode for which 4 different lasers are available (405 nm, 473 nm, 532 nm, 637 nm). Different filter sets allow the simultaneous recording of two fluorescence channels. To allow the generation of image stacks along the z-direction ('z - stacks'), the objective is mounted on a piezo-stage which can be shifted with sub-micron accuracy in z-direction. The fluorescence signal is recorded with an emCCD camera (Andor iXon). For a conventional CCD camera the weak fluorescent light is barely detectable. Therefore an "electron multiplication" camera is used (hence "emCCD"), which multiplies the electrons stored in each pixel prior to the read-out of the camera.

Fluorescent dyes

Fluorescent dyes are used to label cellular structures and to investigate them with the fluorescence microscope. There are fluorescent dyes for various applications. In this practical course, you will get to know a fluorescent marker that labels the actin cytoskeleton, a fluorescent label which is used as a calcium sensor, as well as a photoconvertible fluorescent protein whose transport can be traced in the cell. The two most important parameters of fluorophores are the excitation and emission spectra. They determine which filters to use in a fluorescence microscope. For the use of fluorescent dyes in an experiment it is important to know in which spectral range the fluorochrome absorbs excitation photons. This information is obtained from the absorption spectrum. The maximum emission of fluorescence light is shifted to the red relative to the respective excitation spectrum (Stokes' rule). The spectral composition of the fluorescent light is characterized by the emission spectrum.

Fluorescent Labeling Methods

There are numerous possibilities how to label cellular structures with fluorescent dyes. The easiest way is the use of dyes that stain specific structures by themselves. An example is the lipophilic membrane dye DiI. Although other substances can also bind specifically to certain structures, they do not fluoresce. To visualize them with a fluorescence microscope, they must be labeled with fluorophores. A well-known example is the fungal toxin phalloidin, which is commonly used to label actin. Since phalloidin does not fluoresce itself, it is coupled with fluorescent dyes (see also the experiment "protein labeling"), in our case with rhodamine (Fig. 4). However, the most common stains are made by using antibodies or fluorescent proteins. The importance of these two methods lies in the fact that they are applicable for almost all cellular structures, especially for marking specific proteins.

In this experiment, the actin filaments of the cytoskeleton from human HeLa cells are visualized by a rhodamine-phalloidin staining approach.

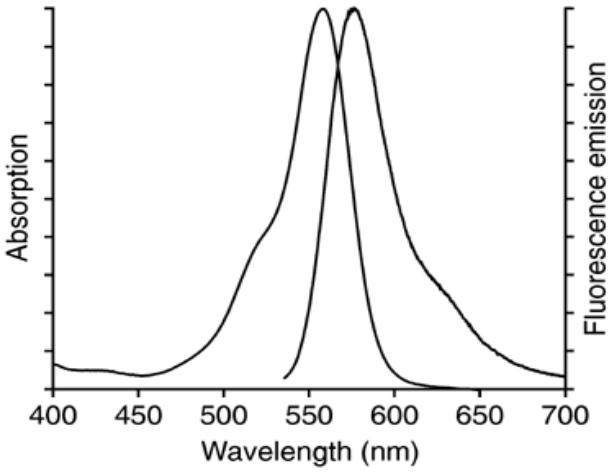


Fig. 4: Excitation- and emission spectrum of rhodamine (Source: www.invitrogen.com)

Antibody staining

Immunostaining is a fluorescent labeling method which uses dye-coupled antibodies. Antibodies are proteins that are able to recognize a specific antigen. The portion of the antigen that is recognized by the antibody is called epitope. If it is possible to couple a dye molecule to the antibody, the dye is also bound specifically to the antigen.

A distinction is made between primary and secondary antibody labeling. The primary antibody labeling strategy is also referred to as "direct method", as the antibody is coupled directly to a dye. The advantage of this method lies in its simplicity. Since only a few steps are required, it is unlikely that artifacts are built.

The secondary antibody labeling method is also called "indirect method", as two antibodies are needed. The first or the primary antibody binds to the antigen; the second or the secondary antibody recognizes the primary antibody species-specific and binds to it. In this case, the secondary antibody is coupled to a dye. The advantage of the indirect method is that several secondary antibodies can bind to a single primary antibody. If only a few epitopes are present, an amplification of the fluorescence signal can be achieved, which is an advantage in comparison to the direct method. In addition, the secondary method is very flexible, as secondary antibodies are usually directed against numerous primary antibodies. This means that their number is manageable and most secondary antibodies are commercially available. But above all, a dye can be exchanged relatively easily by varying the secondary antibody. In the direct method, however, a new labeling process is necessary each time.

Generally, antibody staining is carried out with fixed and thus dead cells. The big advantage is the high achievable specificity. Moreover, almost all types of dyes can be used for this process. This includes not only traditional fluorophores, but also newer developments, such as Quantum dots.

Fluorescent Proteins

An alternative to immunofluorescence represents the transfection of the cells with DNA encoding for fluorescent proteins. Thereby a hybrid of the DNA of a protein, which is to be examined, and the DNA of a fluorescent protein is constructed. This construct is incorporated into a plasmid and introduced into the cell via transfection. The cell then produces the fusion protein. Alternatively, instead of a protein a particular target sequence can be used for the fusion with the fluorescent protein, resulting in a labeling of specific organelles or subcompartments.

The first fluorescent protein (FP), which was successfully used for labeling, was the green fluorescent protein (GFP). It is still one of the most widespread FPs. There is huge number of engineered variants of FPs meanwhile, which span the whole color palette (Fig. 5). These include amongst others yellow fluorescent protein (YFP), blue fluorescent protein (BFP) and red fluorescent protein (RFP). All exist today in an improved form; e.g. eYFP stands for enhanced yellow fluorescent protein.

Fluorescent proteins occur in nature mostly as dimers or tetramers. In transfected samples this may lead to artifacts and to the formation of aggregates. Therefore, monomeric forms of the FPs are constructed, which retain their fluorescent properties (for example: mCherry stands for monomeric Cherry).

The use of FPs has greatly increased in recent years, especially since they offer several advantages over antibody labeling, e.g. for observations in living cells. Thus, not only the spatial but also the temporal distribution of the labeled protein can be observed. In addition, the fluorescent proteins are automatically expressed in the cells without the need for permeabilization or other methods to bring in the dyes from outside.

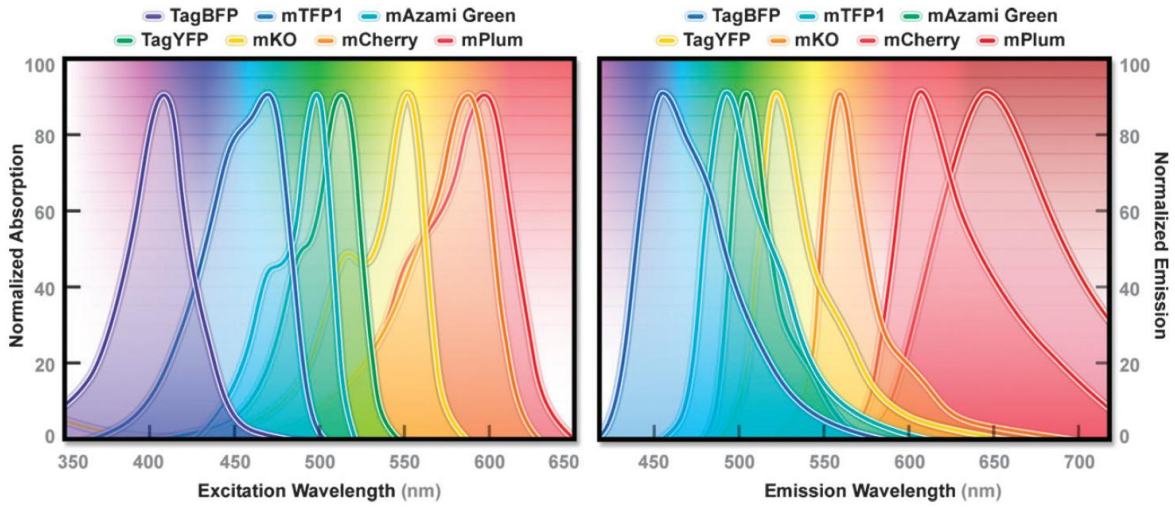


Fig. 5: A color palette of FPs. Absorption and emission spectra of some Anthozoa-derived engineered fluorescent proteins.
(Source: Day,R.N.; Davidson,M.W. Chem.Soc.Rev., 2009, 38, 2887-2921)

Experiments

Rhodamine-Phalloidin Staining of HeLa Cells

In the practical experiment, we will label the actin cytoskeleton of HeLa cells using the rhodamine – phalloidin staining.

Actin is a structural protein, which forms a dynamic network with microtubules and intermediate filaments in the cell: the cytoskeleton. The actin network stabilizes the cell structure and plays an important role in the intracellular transport and during the contraction of muscle cells. Subunits of globular actin (G-actin) can polymerize to filamentous actin (F-actin) and form long actin filaments. These long filaments consist of two actin strands, which are helically wound around each other and which have an overall diameter of 7 nm. Phalloidin is a toxin from the white *Amanita phalloides*, which binds to F- actin with high affinity. It can be coupled to different fluorescent dyes, e.g. to the orange-fluorescent dye tetramethylrhodamine isothiocyanate (TRITC). In nanomolar concentrations the phalloidin staining can be optimally applied to label, identify and quantify F-actin in formaldehyde-fixed and permeabilized tissue sections, cell cultures or cell-free experiments.

Detection of Ca^{2+} Oscillations in HeLa Cells in Response to Various Stimuli

The concentration of free calcium in the cytosol of a cell is a dynamic signal that regulates a wide variety of cellular functions such as secretion, metabolism and transcription. The cytosolic calcium concentration can be increased either by an influx into the cell from the outside or by an efflux from intracellular calcium stores such as the endoplasmic reticulum (ER). To observe the concentration of calcium in living cells, we use the calcium-sensitive fluorescent dye Fluo-4. This calcium indicator undergoes a strong fluorescence increase upon binding to calcium. In its pure form, however, Fluo-4 is negatively charged and cannot penetrate the cell membrane. By esterification with acetoxymethyl (AM) the indicator becomes lipophilic so that it will enter the cell through the membrane. Esterases, which are present in the cytoplasm, remove the ester groups again. Thus, Fluo-4 is an ideal molecular sensor to monitor the intracellular calcium concentrations in living cells.

In the practical experiment, we will observe the change of Ca^{2+} concentrations in HeLa cells in response to various stimuli.

Histamine is a biogenic amine, which is involved in a variety of complex biological processes. By interacting with specific histamine receptors - a class of G-protein coupled receptors (GPCRs), which are present in many cell types - intracellular signaling cascades are triggered. In this case, phospholipase C is activated, which leads to the production of inositol

1,4,5- trisphosphate (IP3) from the substrate phosphatidylinositol 4,5 - bisphosphate (PIP2). IP3 binds to the IP3 receptor (a calcium channel) in the ER, resulting in a calcium efflux from the ER to the cytoplasm. Among other things, a feedback mechanism is thus activated, which causes calcium to be pumped back into the intracellular stores by specific calcium-ATPases. Afterwards the cell is ready for re- stimulation.

Ionomycin is a selective calcium ionophore which causes a rapid efflux of calcium from intracellular stores and a subsequent calcium influx from the extracellular environment. This leads to a maximal fluorescence signal of Fluo-4 and is used in our practical experiments to normalize the fluorescence signal for each individual cell.

EosFP-Mito Green-to-Red Conversion in HeLa Cells

The possibilities of live-cell microscopy ("Live Cell Imaging") were significantly enhanced by the use of FPs that can be manipulated during the experiment. FPs have been found, which can be reversibly switched between a fluorescent and a non-fluorescent state ("photoswitchable": e.g. Dronpa, PAmCherry). There are also "photoconvertible" FPs, which emission can be changed by irradiation with UV light to longer wavelengths. EosFP is such a "photoconvertible" FP (Fig. 6), which shows first green fluorescence (with an emission maximum at 516 nm). After irradiation with UV light (~ 390 nm) it fluoresces in the red (with an emission peak maximum at 581 nm). The UV light causes a break in the peptide backbone in the vicinity of the chromophore and an extension of the conjugated pi-electron system. Proteins fused to EosFP can be expressed in mammalian cells with the preservation of the biological function. Optical switching of EosFP by localized focused UV light is a powerful tool to selectively track the dynamics of biolmolecules in living cells.

In our experiment, a fusion construct of EosFP and a mitochondrial target sequence is used in HeLa cells to monitor the transport of proteins in the mitochondrial network by using live-cell microscopy.

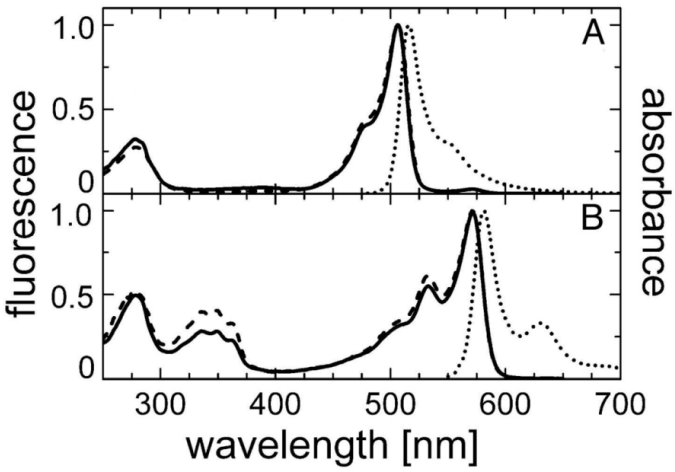


Fig. 6: Absorption and fluorescence spectra of the green fluorescent (A) and red fluorescent (B) form of the photoconvertible protein EOS-FP (Source: Wiedenmann, J. et al. Proc. of SPIE Vol. 6098, 609801 (2006))

Practical procedure

Experiment 1: **Rhodamine-Phalloidin Staining of HeLa Cells**

Materials and Reagents:

- HeLa cells grown on Ibidi®-slides (tissue culture treated 6-channel microscopy slides)
- PBS (Phosphate Buffered Saline, pH ~7.4) (***4°C-box***)
- 4% PFA (Paraformaldehyde) in PBS – take from ***–20°C-box***, keep at 4°C
- 50 mM NH_4Cl in PBS (***4°C-box***)
- Wash Buffer: PBS, 0.01% (wt/vol) saponin, 0.1% Triton X-100 (***4°C-box***)
- Incubation buffer: PBS, 5% (vol) fetal calf serum (FCS), 0.02% (wt/vol) Na-Azide, 0.01% (wt/vol) saponin, 0.1% (vol) Triton X-100 (***4°C-box***)
- Rhodamine-Phalloidin Stock (~ 6.6 µM in Methanol, ***–20°C-box***)
- Ibidi Mounting Medium (***RT***)

1 day before: grow the cells on the Ibidi-slides (0.5 Mio/ml)

Protocol: (use 1 slide of 6 channel Ibidi)

1) Bring 4% PFA to 4°C, PBS – to 37°C, all the rest reagents – to RT
2) Wash the cells **2 times** with **100 µl** of warm (37°C or RT) **PBS**
3) Fix the cells with **100 µl** of cold (4°C) 4% **PFA** in PBS for ***15 min*** (***PFA-waste***!)
4) Wash **2 times** with **100 µl** of **PBS** (***PFA-waste***!)
5) Wash **1 time** with **100 µl** of **Wash Buffer**
6) Permeabilize the cell membranes and block unspecific binding by applying **100 µl** of **Incubation Buffer** for ***30 min***
7) Remove the Incubation Buffer
8) Dilute 2.5 µl of Rhodamine-Phalloidin Stock in 100 µl of Incubation Buffer (1:40 dil.)
9) Add the prepared solution (**100 µl/channel**) to the cells and incubate for ***20 min***
10) Wash **3 times** with **100 µl** of **Wash Buffer**
11) Aspirate the buffer from the channels completely using a piece of tissue paper
12) Add a drop of **mounting medium** to each channel to prevent photobleaching and enable long-term storage of the stained slides
13) *Analyze the stained actin network with a spinning disk confocal fluorescence microscope*
    (532 nm laser ~30%, yellow channel from green-yellow filter cube, use optovar!)
    take single-plane images and z-stacks (d=130 nm, 8 averages) for 3D-reconstruction
    (channel: 'praktikum rho-phall', protocoll: 'praktikum z-stack')
14) *Transform Z-stacks into a **3D view** of the actin cytoskeleton.*
    *Use the '**orthogonal slice viewer**' to observe the actin distribution within the cell*
    *Generate an **animated z-scan** using the 'Movie Editor' (Crop image first)*
    (animation dimension: time, Save list as movie, cinepak codec)

Experiment 2: **Detection of Ca^{2+} Oscillations in HeLa Cells in Response to Various Stimuli**

Materials and Reagents:

- HeLa cells grown on Ibidi®-slides (tissue culture treated 6-channel microscopy slides)
- HBS (Hepes Buffered Saline: 20 mM Hepes, pH 7.4; 143 mM NaCl; 6 mM KCl; 1 mM $MgSO_4$; 5.6 mM Glucose) – stored at 4°C
- HBS-Ca (HBS with calcium: or with 1 mM $CaCl_2$ (prepared from 1M stock) – stored at 4°C
- Fluo-4, AM (2 mM Stock in DMSO) – Ca^{2+}-sensitive fluorescent dye in the form of cell-permeant acetoxymethyl (AM) ester – stored at ***–20°C-box***
- Histamine (100 mM Stock in H_2O) – stored at ***–20°C-box***
- Ionomycin (1 mM Stock in DMSO) – Ca^{2+} ionophore – stored at ***–20°C-box***

1 day before: grow the cells on the Ibidi-slides (0.5 Mio/ml)

Protocol:

1) Bring to **RT**: HBS, HBS-Ca, Put on ice: Histamine (Stock), Ionomycin (Stock)
2) Prepare Epi with 1 ml of HBS
3) Add 1 µl Fluo-4 (Stock) and mix properly
4) Wash the cells in one channel with **100 µl** of **Fluo-4**-containing solution
5) Add **100 µl** of **Fluo-4**-containing solution. Keep for **30 min** at RT (loading)
6) Dilute Histamine Stock 1:100 with HBS (1 µl + 99 µl) → 1 mM Histamine
7) Wash cells in the prepared channel **2 times** with **100 µl** of **HBS** (**Ca^{2+}-free**!)
8) Bring the slide to the microscope and **remove HBS** from the prepared channel
9) *Focus on a group of cells in 'Live'-mode (473 nm laser ~15%, green channel from green-yellow filter cube, no optovar!)*
10) *Start recording the Fluo-4 signal in the cells (run 'DT40' timelapse protocol, 4 averages)*
11) Prepare individual solutions a), b), c) **freshly before each application!** (start with b):
    a. 2 µM Histamine: 1 µl of Histamine (1 mM) in 499 µl of HBS (1:500 dilution)
    b. 10 µM Histamine: 2 µl of Histamine (1 mM) in 198 µl of HBS (1:100 dilution)
    c. 50 µM Histamine: 10 µl of Histamine (1 mM) in 190 µl of HBS (1:20 dilution)
12) Add **100µl** of one of the **prepared solutions** to the channel (note the movie *frame number*)
13) Observe the Ca^{2+}-response to the stimulus
14) In the meantime prepare 5 µM Ionomycin:
    Dilute 2 µl of Ionomycin (1mM) in 398 µl of HBS-Ca (!)
15) Remove solution and add **100 µl 5 µM Ionomycin** to the cells and record the maximal signal from Fluo-4 due to extracellular Ca^{2+} entering the cells

Note: *while doing an experiment on one channel we can **load** the next one with **Fluo-4***

16) *Select specific areas in the cells of interest and perform the mean intensity analysis of the individual areas as a function of time (frame number)*
17) *Generate a movie of the Ca^{2+}-response using the 'Movie Editor' (animation dimension: time, Save list as movie, cinepak codec)*

Experiment 3: **EosFP-Mito Green-to-Red Conversion in HeLa Cells**

Materials and Reagents:

- HeLa cells grown on Ibidi®-slides and expressing EosFP-Mito fusion construct

1 day before: grow the cells on the Ibidi-slides (1 Mio/ml) and transfect with EosFP-Mito-pcDNA3 plasmid coding for EosFP protein primed to the mitochondria

Protocol:

1) *Bring the slide to the microscope, search for an appropriate cell with sufficient expression of the fluorescent protein and observe the mitochondrial network (green detection channel, blue laser 473 nm ~15%). Generate a snapshot image (128 averages).*
2) *Use both channels of the green-yellow filter cube and adjust the image-splitter to generate a dual color image on the camera (side-by-side). Reduce the bleed-through into the yellow/red channel by introducing an additional longpass filter (LP590).*
3) *Switch on the green laser (532 nm laser, ~25%), no mitochondria should be visible in the yellow/red channel.*
4) *Start recording a timelapse movie (run protocol Eos_conv, 4 averages) and switch the **"UV"-laser** on for a short time (~ **1 second**) to identify the target area of the UV-laser. Stop recording after a few seconds and use the movie to mark the **target area** of the UV-laser as a 'region of interest' in the green channel image.* ROI
5) *Find a new cell and place the end of an elongated mitochondrion at the target area of the UV-laser. Start recording a timelapse movie (run protocol Eos_conv, 4 averages) and induce after some time **local conversion of EosFP** (observe the decrease of fluorescence in the green channel and local **increase** and **spreading** of fluorescence in the **red** one).*
6) *Select specific areas in the same cell, pairwise in the green and the red channels (at the point of conversion and at a distant point, respectively) and perform the mean intensity analysis of the individual areas as a function of time (frame number).*

   Note the increase in the red fluorescence and the decrease in the green one after the flash
7) *Find a new cell with sufficient expression of the fluorescent protein. Switch the **"UV"-laser** on for a short time (~ **1 second**) to locally induce **conversion of EosFP** from green to red form.*

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Experiment 2

Video N° 3

from frame number 550 it's LONG MYCIN for the Same

200 ms of Exposure time

-Red = Background

- 3rd Video
  - ↳ dip begin because lost of focus
  - ↳ then we lost the Region because I bushed it!

1 page INTRO
- ↳ Context
- ↳ TOPIC - Related

Cite the Script to Methods §

Experiment
- ↳ Short description of Experiment (AIM)
- ↳ Results
- ↳ Discussion

x3

Exactly 1 week