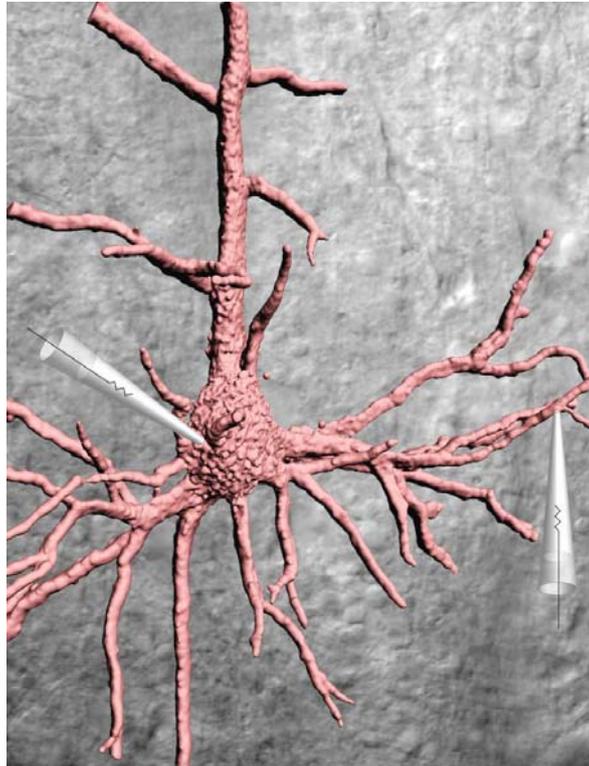


The Leica TCS SP5 DM6000 CFS

Electrophysiology and Deep Tissue Imaging



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Title Page:

3D-reconstruction of a mouse pyramidal neuron in layer 5 filled with Alexa-594. The rendering has been performed by Imaris software and later on combined with the scanning gradient contrast image.

Courtesy of:

Dr. Thomas Nevian, Inst. of Physiology, University of Bern, Switzerland

1. Patch clamping - A main method in electrophysiology

The function of nerve and muscle cells relies on ionic currents flowing through ion channels. These ion channels play a major role in cell physiology. One way to investigate ion channels is to use patch clamping. This method allows for investigating ion channels in detail and to record electric activity of cells.

The patch clamping technique was developed by Erwin Neher and Bert Sakmann in the 1970s and 80s to study individual ion channels in living cells. In 1991 they received the Nobel Prize for Physiology and Medicine for their work. Today the patch clamping technique is one of the most important methods in the field of electrophysiology.

The basic principle of patch clamping:

A tight seal is formed between a glass pipette and the cell membrane. This arrangement allows for the recording of small currents and voltages involved in the activity of neurons and other cells.

- A glass pipette with an open tip ($\sim 1 \mu\text{m}$) is used. The interior of the pipette is filled with solutions and an electrode. The metal electrode in contact with the solution conducts electrical changes to a voltage clamp amplifier. In this way changes in ion flux through the channel can be measured.
- Pressure is applied to the pipette in order to penetrate through the tissue to the cell of interest.
- Once a cell of interest is identified, the patch clamp pipette is pressed against the membrane, and suction is applied to pull the cell's membrane inside the tip (Fig. 1 a). The suction causes the cell to form a tight seal with the electrode, the so-called giga-seal (high resistance between the pipette and bath solution in the range of gigaohms) (Fig. 1 b).
- A brief suction disrupts the membrane patch below the pipette and an open conduit from the pipette to the lumen of the cell is formed. This is the so-called whole-cell configuration. The patch clamp technique can be applied in multiple configurations (Fig. 1 c)

The composition of solutions can be customized for the application; drugs may also be added. Patch clamping experiments can be performed on different types of cells, mainly excitable cells like neurons, muscle fibres or beta cells of the pancreas.

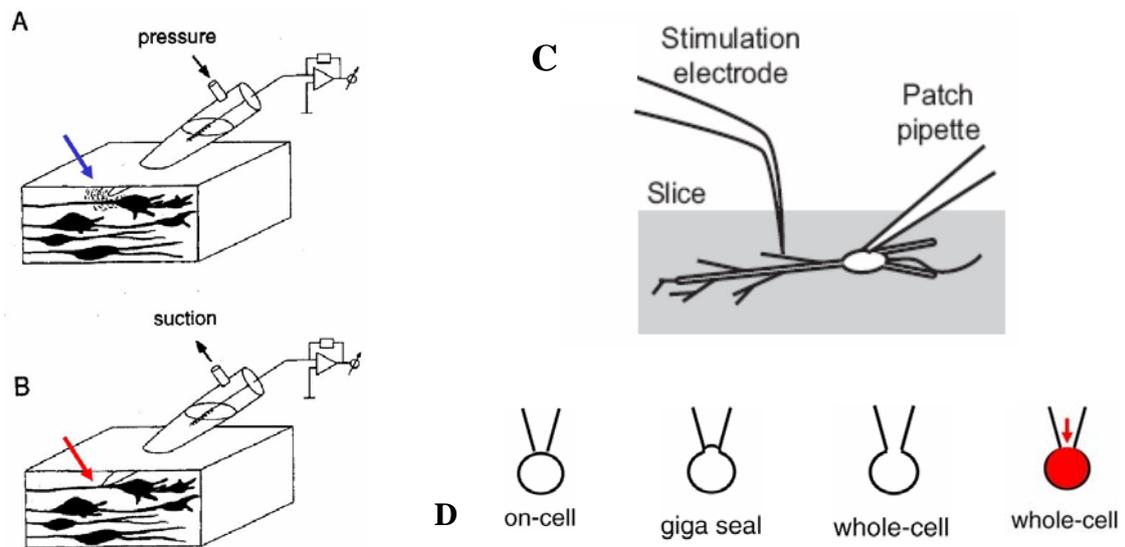


Fig. 1: the principle of the patch clamp technique. a) Pressure is applied to the pipette to penetrate through the tissue. b) Suction is applied to form a tight contact with the cell. (a) and b) adapted from Stuart et al. 1993) c) A patch electrode is attached to a neuron to record voltage signals. A stimulation electrode can be placed close to a dendrite for extracellular stimulation by voltage pulses. d) Steps to get a whole cell configuration: the soma of the cell is approached (on cell), formation of a giga-seal by releasing pressure, brief suction disrupts the membrane (whole cell) cell can be filled with dyes or drugs. d) from PhD-thesis of Th. Nevian: "Calcium dynamics in dendrites and spines in the somatosensory "barrel" cortex of the rat", 2003.

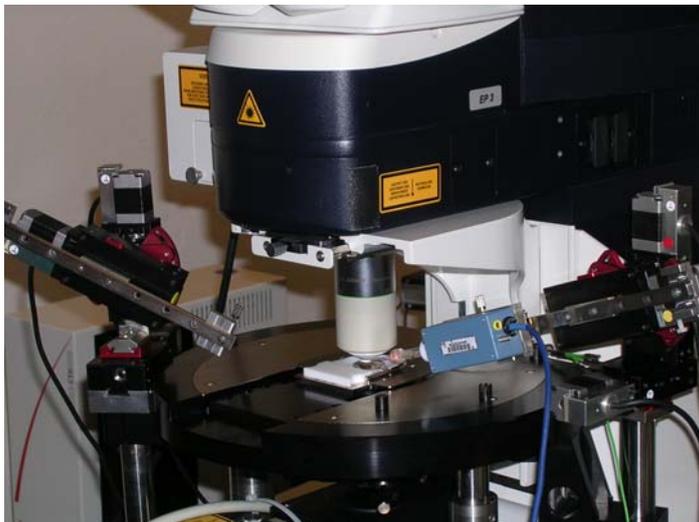


Fig. 2: Setup of a Leica DM6000 CFS: A micromanipulator is located close to a brain slice, which is placed in a mini chamber on the platform of the fixed stage.

2. Changing between low and high magnifications without changing the objective

In patch clamp experiments a large observation field is necessary in order to find the regions of interest within a specimen for example in the brain of a living mouse, living brains slices, embryos, etc.. In addition, it is important to be able to visualize detailed structures so that pipettes can be placed accurately.

The Leica DM6000 CFS combines the new high NA/low magnification objective lens (HCX APO L 20x/1.0W) with the magnification changer in the CCD-camera mode. This combination allows for a fast overview of the specimen while keeping a large observation field (Fig. 3). Furthermore, the observation field can be changed easily by using different magnification changers.

A large observation field is critical to accurately position pipettes and electrodes in the specimen. This large field of view is achieved using the 20x objective. To identify individual cells, the 4x magnification changer can be used. Therefore, using both the objective and the magnification changer it is possible to easily position pipettes very close to the cell. If a higher resolution is required, as for the investigation of neuronal spines, the system can be switched to confocal scan mode.

Since objectives do not need to be changed, vibrations are minimal, and the specimen and manipulators are maintained in their proper position.

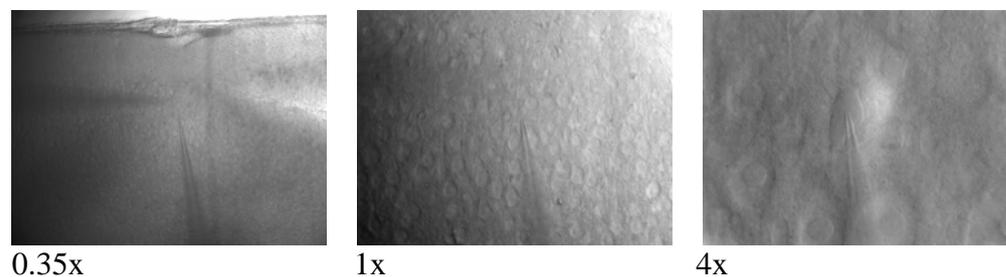


Fig. 3: Identification of cells: Dot gradient contrast transmitted light images of a mouse brain slice taken by a CCD-camera (Leica DFC 360FX). To identify the layer of interest in a brain slice, overview imaging was performed using the magnification changers 0.35 and 1.0. Single cells were selected for the experiment when using the 4x magnification changer.

3. Rapid loading of neurons with dyes by single cell electroporation

To study neuronal networks in the brain, specific labeling of individual cells is required. A new method, called single cell electroporation (Nevian and Helmchen 2007), allows for a rapid and selective loading of cells.

Principle:

Brief voltage pulses are delivered causing an electromagnetic field is applied to the membrane. This results in the transient formation of small pores; the pores close within seconds. Charged molecules are transported in the direction of the electrochemical gradient during opening of the pores. This method can be used to load neurons with calcium-sensitive dyes and other dyes (Fig. 4 a and b).

Using the single cell electroporation technique, the background staining is lower compared to using the loading by intracellular recording electrodes or patch pipettes. The single cell electroporation technique is especially well suited for functional imaging of subcellular Ca^{2+} -dynamics *in vitro* and *in vivo*. Multiple substances can be loaded to obtain morphological and functional measurements at the same time. Furthermore, multiple cells can be loaded sequentially in order to image small neuronal networks by using the same pipette.



Fig. 4 a

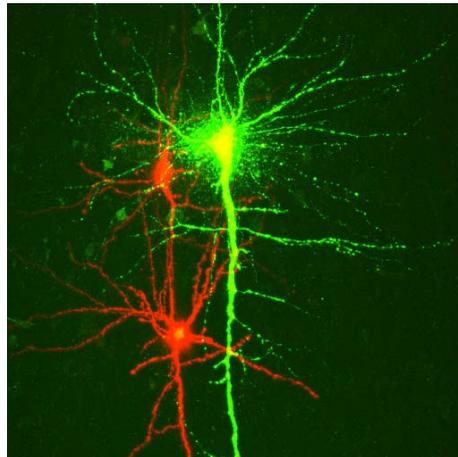


Fig. 4 b

Fig. 4: (a) A specific neuron was loaded with a calcium sensitive dye by single cell electroporation; overlay of simultaneous acquisition of fluorescence with a Non-Descanned Detector (NDD) and transmitted light; (b) small neuronal network: rat brain slice, layer 5, red: Interneurons Alexa 594, green: Pyramidal Cell Oregon Green Bapta 1 (calcium sensitive), $Z = 123\mu\text{m}$, two-photon excitation; detection with a 2-channel NDD.

Courtesy of: Dr. Thomas Nevian, Inst. of Physiology, Univ. of Bern, Switzerland



Specifications of the Leica HCX APO L 20x 1.0W

- Low magnification, 20x
- High resolution with a high NA 1.0
- 2 mm free working distance
- Access angle 39° for good sample accessibility
- Ceramic non-conducting lower part for interference-free patch clamping
- High VIS-IR transmission:
VIS 85% - IR 90% @ 800nm

4. High resolution imaging with the new Leica objective HCX APO L 20x/1.0 W and the SP5 scanner

For many physiological applications it is necessary to image with a maximum field of view and with the highest resolution. The highest resolution of the new objective lens HCX APO L 20x/1.0 W is 195 nm in the xy-dimension. It allows for imaging the smallest details at zoom factor 1 when scanning with the highest pixel format.

Example:

The image size in the object plane with the HCX APO L 20x/1.0 W lens at zoom 1 is 775 μ m. The maximum xy-resolution at 488 nm is 195 nm. According to the Nyquist-sampling criterion, a pixel needs to be approximately 100 nm in size to ensure the maximum resolution is obtained. To achieve a 100 nm pixel size over 775 μ m length, about 8000 pixels per line are required. This is easily accomplished using the 8192 x 8192 pixel format of the SP5 for scanning (Fig. 5). As a result, overview images and high resolution images can be acquired without changing the objective lens.

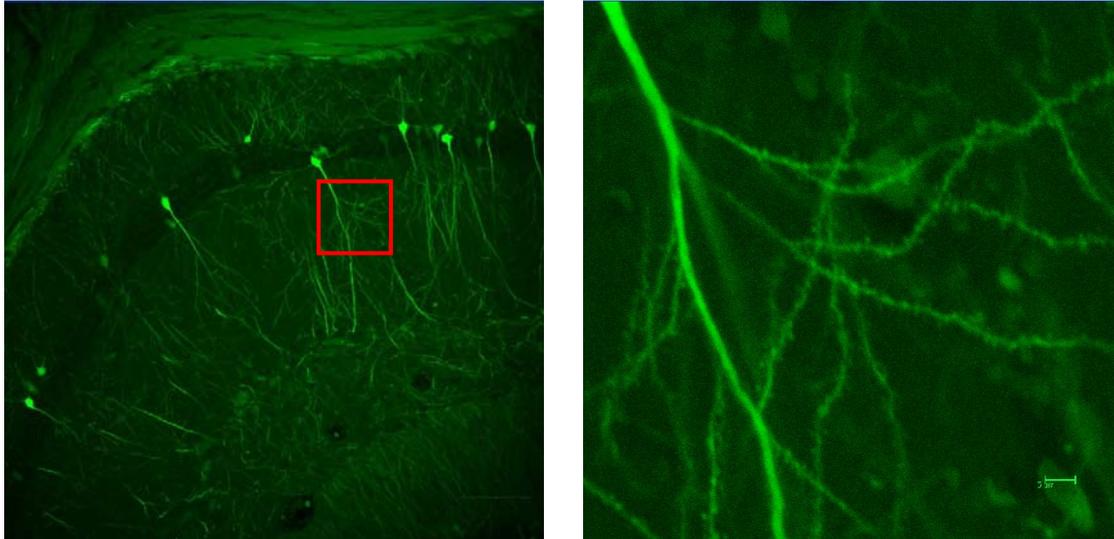


Fig. 5: Mouse brain slice, GFP expression in specific neurons. Image acquired with highest pixel format 8192 x 8192 at zoom factor 1, objective used: HCX APO L 20x/1.0 W. Overview image (left). Upon zooming in on the display small details like neuronal spines are visible (right image, bar 5 micro meters).

5. Correlation of optical and electrical data - and images

In many physiological applications the reaction of cells to different types of stimuli is of interest. The reactions of the cells, as seen by electrical and fluorescence intensity data, need to be measured and displayed in a synchronized manner. The intensity data usually refer to the intracellular calcium concentration or pH-value.

The new Leica DM6000 CFS enables the synchronized correlation of electrical and optical data; voltage recordings are correlated in synchronization with the fluorescence intensity data and are automatically displayed in graphs. This reveals a fast and direct overview of the experimental progress and online data evaluation. Furthermore, images are displayed below the graphs to get fast information about the morphology of the cell. A data acquisition box and Leica trigger unit are integral components for viewing the intensity- and voltage data correlation.

Recorded signals, as from a neuron, are typically amplified, and then sent to a NI Data Acquisition Box (DAQ box) from National Instruments, which digitalizes the signals. The DAQ-box is connected to the trigger unit that allows for the synchronization with the scanning process. It is also connected to the PC so that correlated optical and electrical data, as well as images, can be displayed (see Fig. 6).

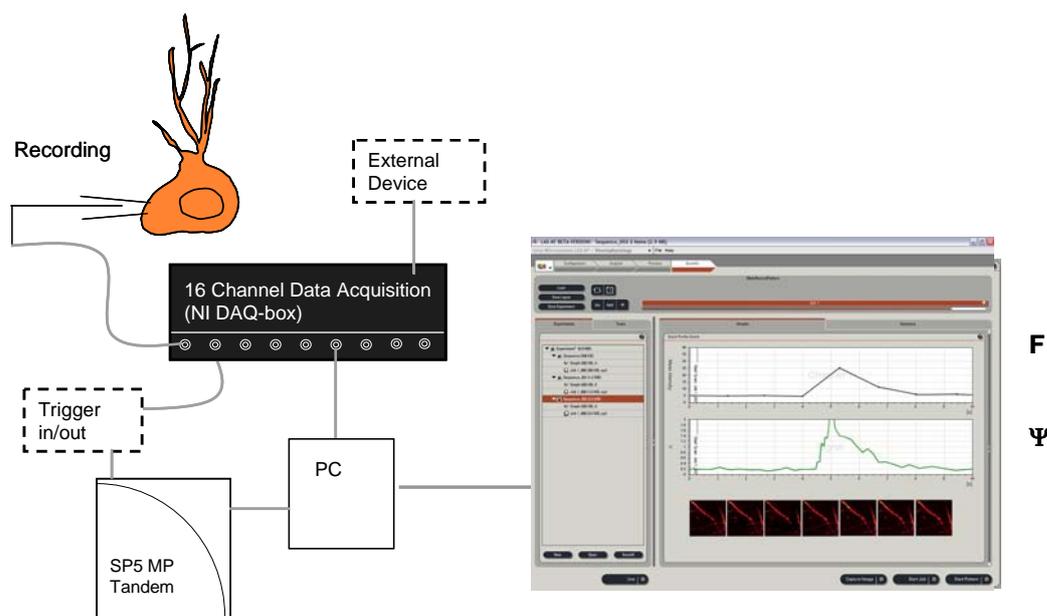


Fig 6: Scheme of connected components for correlation of optical data, electrical data, and images. An external device can be any instrument like a patch clamp setup, a pulse generator, a pump, etc.

6. Currents and calcium in heart muscle cells

Isolated cardiomyocytes from trout are labeled with the calcium-sensitive dye Fluo4. They are stimulated by a trigger pulsing regime using patch clamping (HEKA EPC-10 double). Cardiomyocyte responses to intracellular calcium concentration and ionic currents are measured using fluorescence imaging and electrical recordings. The stimulation protocol on the patch clamp setup was synchronized with the confocal time lapse series using a trigger on the patch clamp setup to mark events in individual frames on the time axis (Fig. 7 a).

Line triggers are recorded in order to have the exact correlation of the image scan, fluorescence signal intensity, and electrical response of the cell (Fig 7b-d). These triggers are automatically generated by the scan head. This means that whenever a line is scanned, its corresponding trigger pulse is recorded and displayed in the quantification chart. Synchronization of images and triggered pulses is accomplished using the Leica DAQ box.

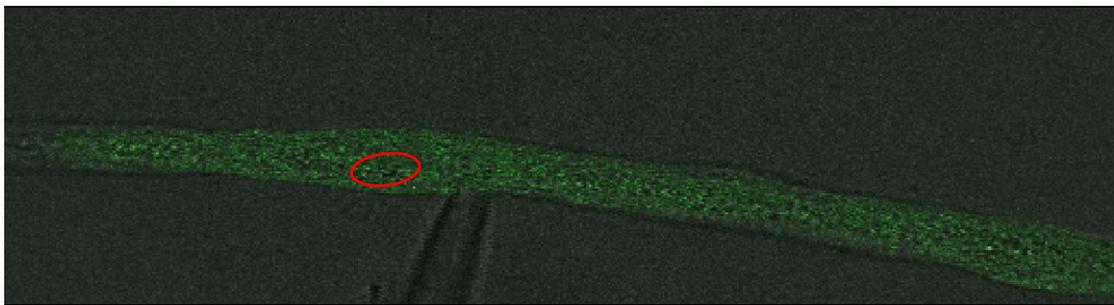


Fig. 7 a: Heart muscle cell (cardiomyocyte) with attached patch pipette. Overlay of fluorescence (Fluo4) and transmitted light channel. Fluorescence signal measurements were taken inside the ROI.

Courtesy: Leif Hove-Madsen, Catalan Cardiovascular Institute, Hospital San Pau, Barcelona, Spain.

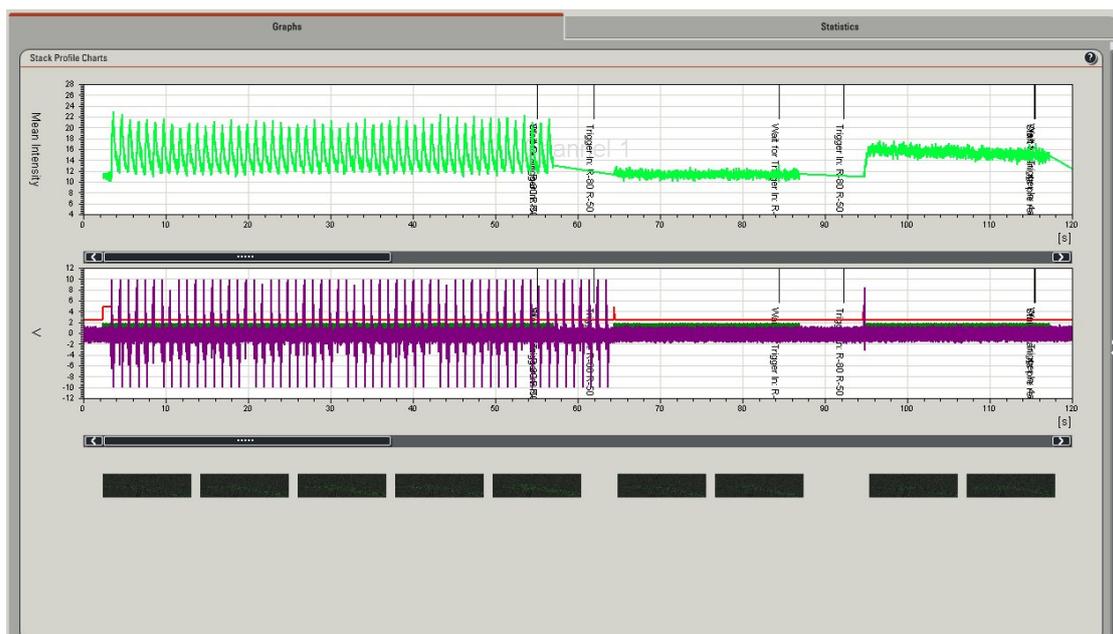


Fig. 7 b: Quantification of the experiment described in the text. Intensity graph (top), electrical data (middle) and images (bottom) recorded during a patch clamp experiment in a cardiomyocyte. Cells have been stimulated by different pulsing protocols, and their reactions as indicated by ionic currents and calcium signal intensity were measured within an ROI. Current recorded (purple), trigger pulses (red) and line trigger (green).

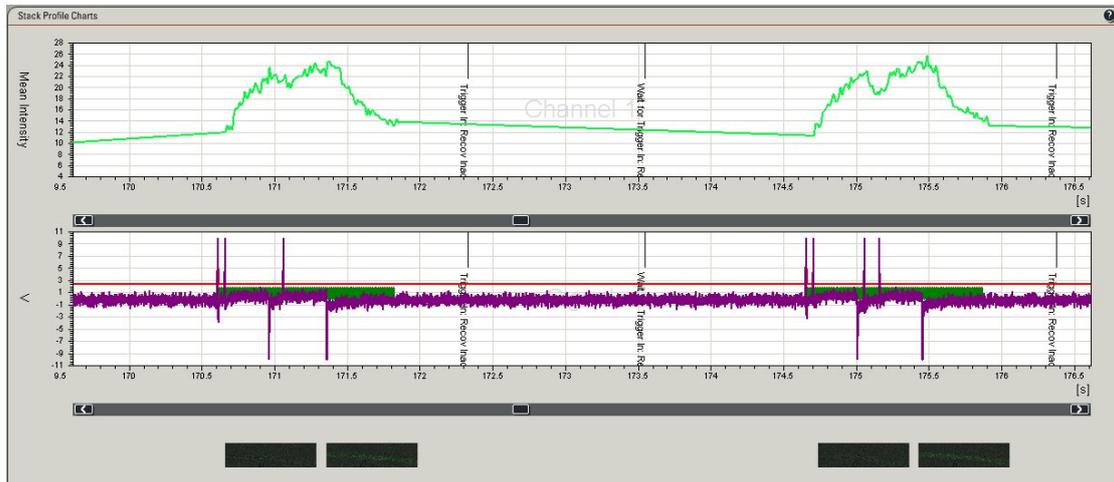


Fig. 7 c: Details from the same experiment as described for Fig. 9a and b: Intensity graph (top), electrical data (middle) and images (bottom). Electrical data showing the current recorded (purple), trigger pulses (red) and line trigger (green). Note that the signal intensity peaks correspond perfectly with the recorded line triggers and the images.

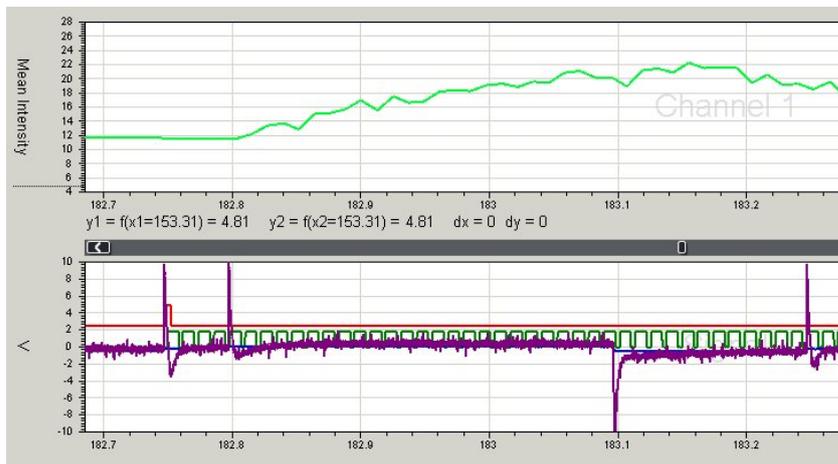


Fig. 7 d: Upon zooming into the graph the individual line triggers (green) are visible.

7. Using Multiphoton Microscopy and external detectors for deep tissue imaging

A major challenge in physiological applications is the study of cells in deeper layers of tissue. Multiphoton microscopy provides several advantages to solve this challenge. Lower light scattering, restricted excitation and bleaching to the focal plane, and reduced phototoxicity are the properties of multiphoton microscopy that enable visualization of deep structures.

Brain slices, for example, are highly scattering and are usually sliced several hundred micrometers thick. Therefore it is very difficult to image brain slices using a standard confocal microscope. Thicker specimens can be imaged using multiphoton microscopy due to an enhancement in photon collecting efficiency.

In confocal microscopy the pinhole aperture rejects the out-of-focus fluorescence light as well as the scattered (diffused) light. Since the scattered light is not 'seen' by the detector, it is not easy to image highly light scattering tissues such as thick brain slices.

In multiphoton microscopy however, no confocal pinhole is required to reject the out-of-focus light because all fluorescent light originates from the focal spot. The emitted light from the specimen does not need to pass through the microscope again. Therefore, detectors can be placed as close as possible to the specimen so that scattered photons can be collected. In this way significantly higher photon collection efficiency is achieved compared to confocal microscopy (Fig. 8).

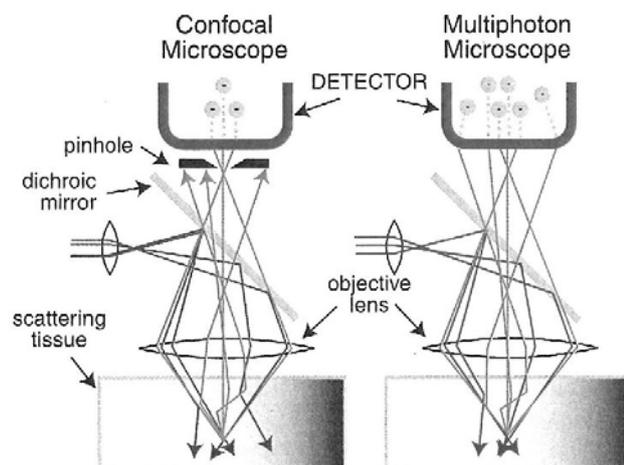


Fig 8: Difference between confocal and multiphoton microscopy, Denk, 1996

The external Non-Descanned Detectors (NDDs) are PMTs for collecting emitted light from the focused and scattered light.

The NDDs are boxes containing PMTs for the detection of either two or four different fluorescence signals (4-channel NDD). To separate the signals, several filter cubes are available and can be introduced into the detector-box (Fig. 9).

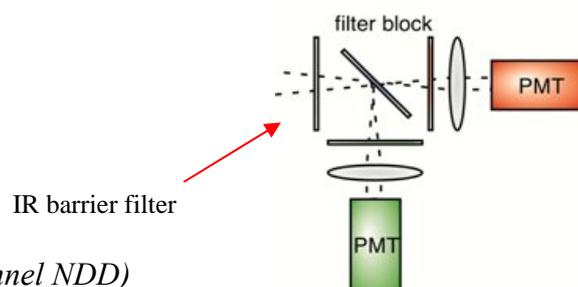


Fig. 9: Inside an NDD-box (2 Channel NDD)

2-channel and 4-channel external detectors (NDDs):

Two pairs of NDDs can be attached to the microscope: the NDD-RLDs (Reflected Light Detectors) and the NDD-TLDs (transmitted light detectors). Alternatively, a 4-channel NDD detector can be used to collect either reflected or for transmitted light. In this case the light is guided from the objective directly to the external detectors by a fiber.

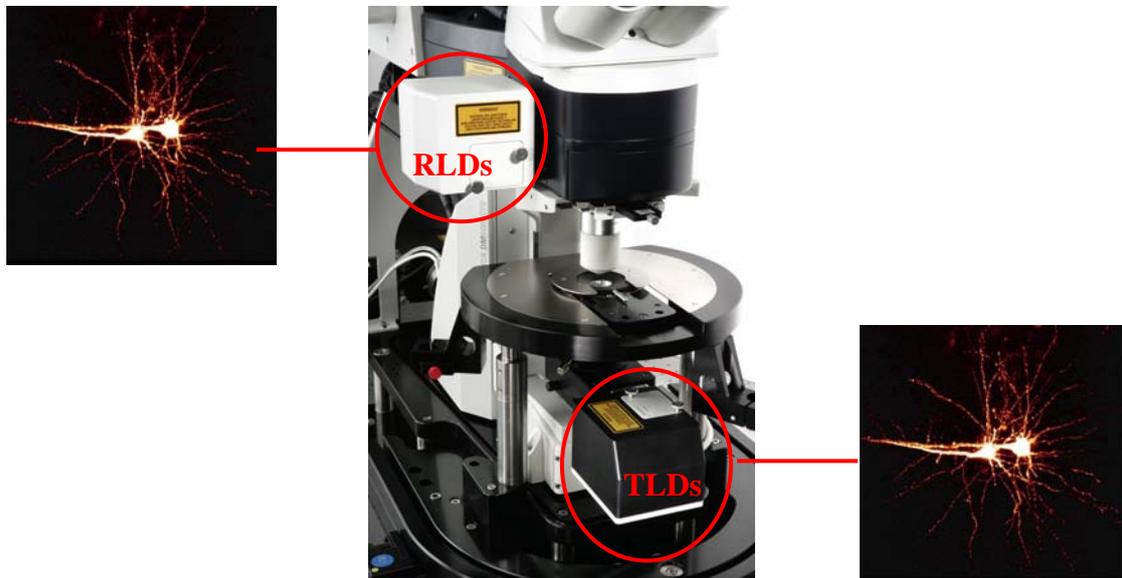


Fig. 10: A Leica DM6000 CFS System equipped with external detectors (NDDs). Images: neurons selectively marked in a 300µm thick living mouse brain section excited with IR-light.

Two neurons in a living brain slice labeled with Oregon Green Bapta 1, two-photon excitation, detection of the same fluorescence by RLD and TLD.

Courtesy of: Dr. Thomas Nevian, Inst. of Physiology, Univ of Bern, Switzerland

NDD-RLDs

Upon multiphoton excitation the RLDs are used to detect fluorescence coming from a sample that is not transparent at all, like imaging in the living brain or other organs. A Reflection Short Pass filter (RSP), a 670 filter, is placed in the fluorescence cube turret. This filter allows passage of the two-photon-excitation (IR) to the specimen. The emitted light from the specimen (in the VIS-range) is reflected to the detector (wavelengths shorter 670 nm).

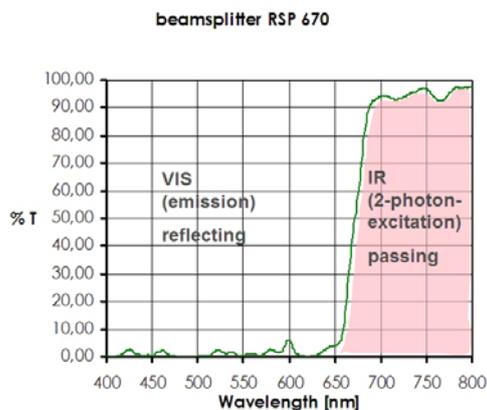


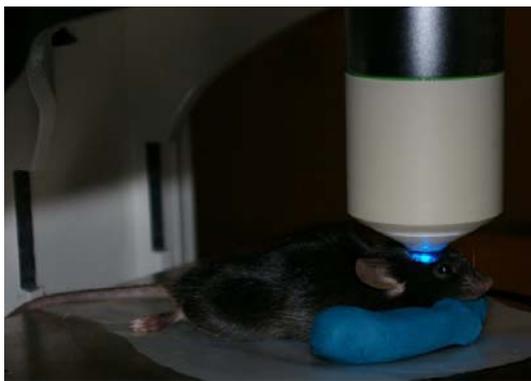
Fig. 11: Spectral properties of RSP 670

NDD-TLDs

For imaging transparent samples, like tissue slices or cultured cells, the TLDs can be used for detecting fluorescence below the sample. Furthermore, the TLDs can also be used for imaging the second harmonic generation (SHG). In this case, a high numerical aperture oil-condenser (e.g. NA 1.4) should be used. This enhances the capability of collecting light, and therefore enhances the photon collection efficiency.

In vivo Imaging

For investigation of live animals and deep imaging it is crucial to match the refractive indices of the medium and the intermedium. For example, live brain tissue needs to be immersed in a water-like physiological solution to keep it alive, so it is mandatory to use a water immersion lens for imaging. The goal is to maintain the water column between the brain and the objective front lens. Therefore, stable focus conditions during imaging are required, and the animal's head must be stabilized. The head can be stabilized by using modelling clay (Fig. 12a) or a mouse head holder that allows for very precise adjustment of the mouse head (Fig. 12b).



a



b

Fig: 12 a: Modelling clay for fixing the head of a mouse; b: mouse head holder from Luigs & Neumann

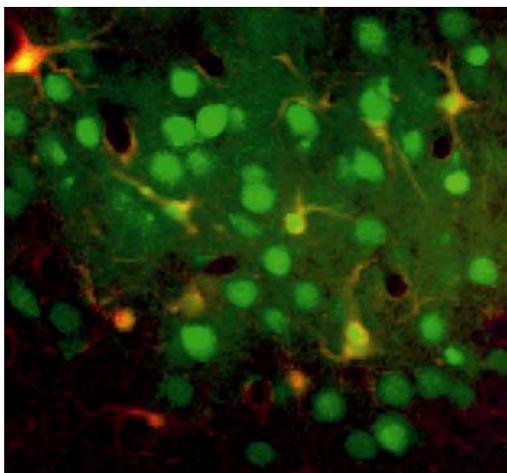


Fig. 13: Living mouse brain, double staining with Oregon Green and Sulfo-Rhodamine-101, ca. 300 μ m inside. Courtesy: Jason Kerr, Max Planck Institute for Medical Research Heidelberg, Germany.

Deep imaging of Mouse Tissue with Multiphoton Microscopy

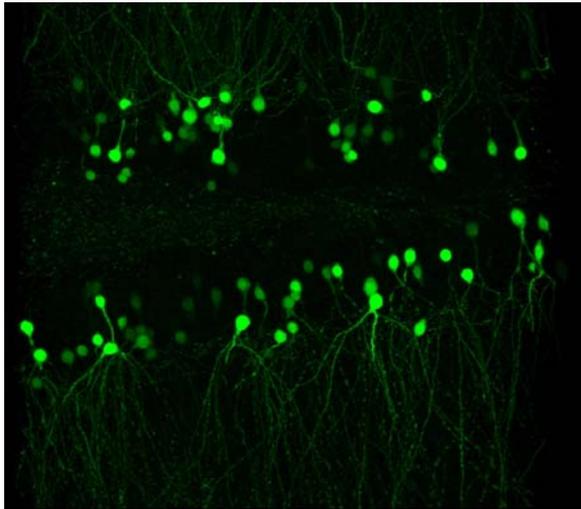


Fig. 14: Acute mouse brain section containing YFP neurons. Maximum projection of an xyz-stack.

Courtesy of Dr. Feng Zhang; Deisseroth Laboratory, Stanford University, USA

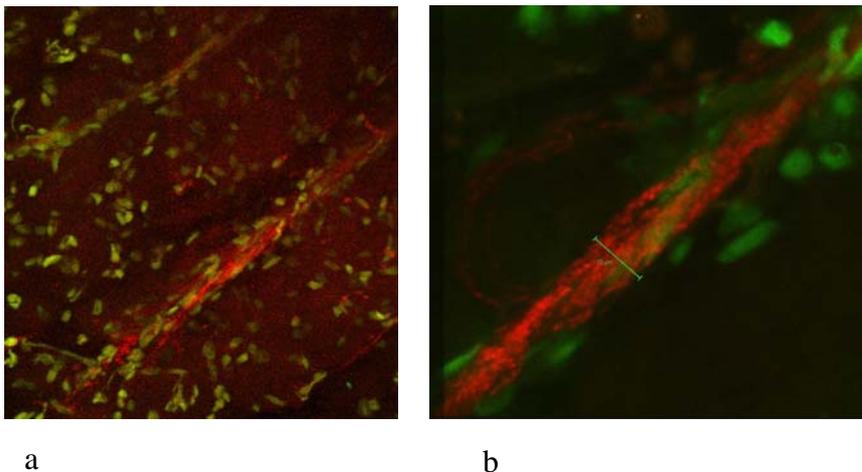


Fig.15: Mouse heart: The heart was excised and a 2% agarose gel was subsequently cast around a 1mm thick slice of the organ. Two-photon excitation was used with 840 nm . Fluorescent labeling of cell nuclei was done with Syto13 (green).s. a) Overview image, cell nuclei of cardiomyocytes are visible. The “threads” of nuclei belong to endothelial cells in the capillaries around the cardiomyocytes. In red the second harmonic generation signal of collagen in the capillaries is visible. The vague reddish background is autofluorescence of the cardiomyocytes. b) Zoomed image, second harmonic generation signal of collagen and its association with endothelial cells is more clearly visible.

Courtesy of Marc van Zandvoort, Biophysics, Univ. of Maastricht, Netherlands.

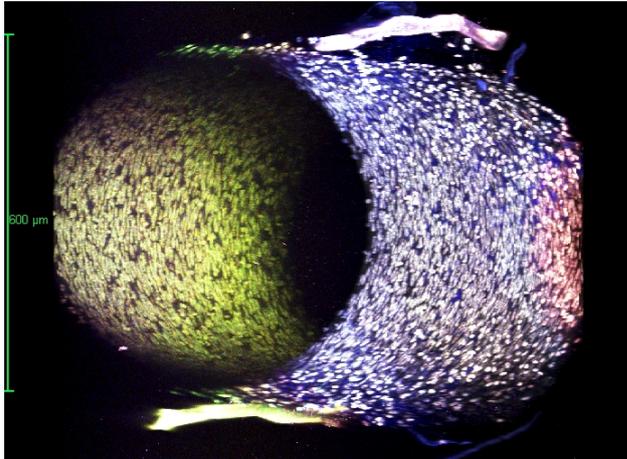


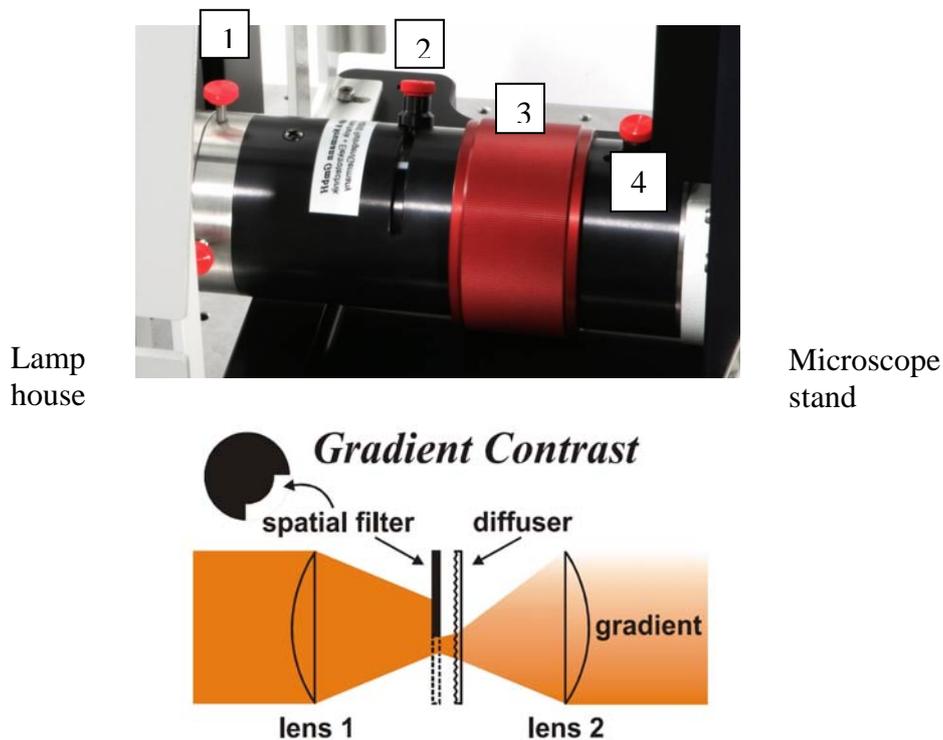
Fig. 16: Artery of the mouse: Excitation at 890nm, 3-channel acquisition: autofluorescence of elastin (blue), Syto13 for nuclei of cells in the vascular wall (green/white), eosin auto-fluorescence (red); zoom 1. Imaging depth 650μm. Preparation: Common carotid arteries from mice are carefully dissected, excised, and stored in Hanks' balanced salt solution (HBSS, pH 7.4). A single artery is mounted on two glass micropipettes in a homebuilt perfusion chamber and perfused gently with HBSS to remove residual luminal blood. The distance between the pipettes can be modified to adjust for shortening of the arteries during the isolation process. Afterwards, a transmural pressure of 80 mmHg is applied to mimic a more physiological situation. Courtesy of Marc van Zandvoort, Biophysics, Univ. of Maastricht, Netherlands.

8. Simultaneous acquisition of fluorescence and high contrast transmitted light imaging (Dodt Contrast)

How to visualize unstained cells in thick tissue?

Unstained neurons in thick brain slices are phase objects. To make them visible it is necessary to convert their phase gradients into amplitude gradients. There are different ways to achieve this. One is by using differential interference contrast (DIC), which requires prisms and polarization filters in the beam path. Thus, when transmitted light and fluorescence are imaged together the photon collecting efficiency is reduced due to the optical components in the beam path.

Infrared Scanning Gradient Contrast (SGC-Imaging) – also referred to as Dodt Contrast – is another method used to visualize unstained cells in thick scattering tissue. This is a special optical system developed by Hans-Ulrich Dodt (Max-Planck Institute, Munich). In this technique the contrast can be specifically adjusted to enable different structures (horizontal or vertical) to be highlighted.



*Fig.17: Dodt contrast tube and scheme of the Dodt contrast tube inside: The Dodt contrast tube is mounted between the microscope and the lamp housing
 Knob 1 is for connecting the tube to the lamp house.
 Knob 2 is for altering the direction of the spatial filter that manipulates the direction of contrast of the image.
 Upon turning the red zoom ring (3) the diffuser position in z (optical axis) is changed. This influences the strength of contrast of the image.
 With knob 4 one can position a lens according to the condenser lens used (for NA 1.4 closest to the microscope, for NA 0.9 farthest from the microscope).*

Working Principle: Oblique detection and Scattering

Using the Dodt gradient contrast method no optical components are needed in the beam path to get high-contrast and high-resolution images. Here, a lens system between the microscope stage and the lamp house re-images the aperture plane of the condenser. Spatial filtering is performed by a quarter annulus located in the illumination beam. After the annulus, a diffuser is placed to generate an oblique illumination across the condenser aperture. The light-stop blocks much of the illuminating light and only a part of the illuminating light cone is used. Therefore, less stray light is generated in the slice and an oil-immersion condenser, e.g. with 1.4 NA, can be used for high resolution images. Adjustments can be made to the direction of the spatial filter and to the distance of the diffuser with respect to the spatial filter (see description for Fig. 17).

The contrast created is very strong and images look similar to DIC-images. The Dodt gradient contrast images are detected with a transmitted light detector or so called Dodt-detector. The PMT of the Dodt-detector has a higher IR-sensitivity (up to approx. 900 nm) compared to regular PMTs. A CFS-system equipped with NDDs and a Dodt-detector allows for simultaneous detection of fluorescence when using IR-excitation (Fig. 18). It provides the highest photon collection efficiency as there are no optical components in the beam path. This also facilitates many applications in physiology, e.g. optically guided patch clamping (Fig. 19).

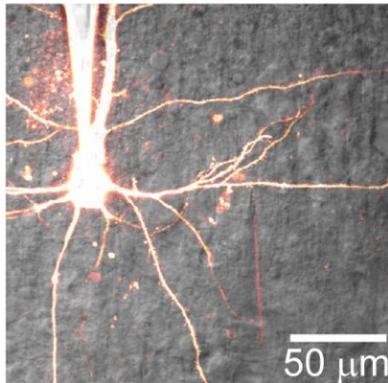


Fig. 18: Overlay of simultaneous acquisition of fluorescence with RLD (NDD) and transmitted light using the Dodt-detector and IR-excitation; this enables optically guided patch clamping in living brain slices.

Courtesy: Thomas Nevian, Inst. Of Physiology, Univ. of Bern, Switzerland

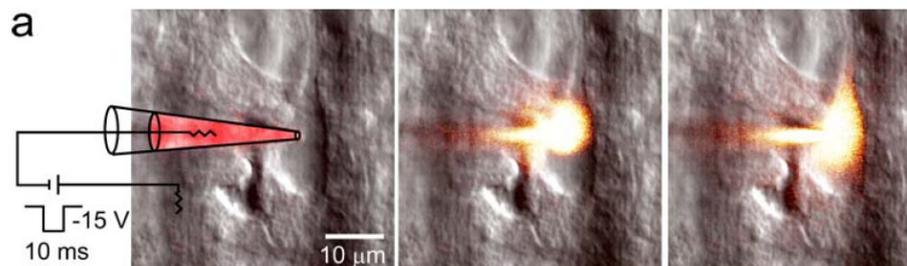
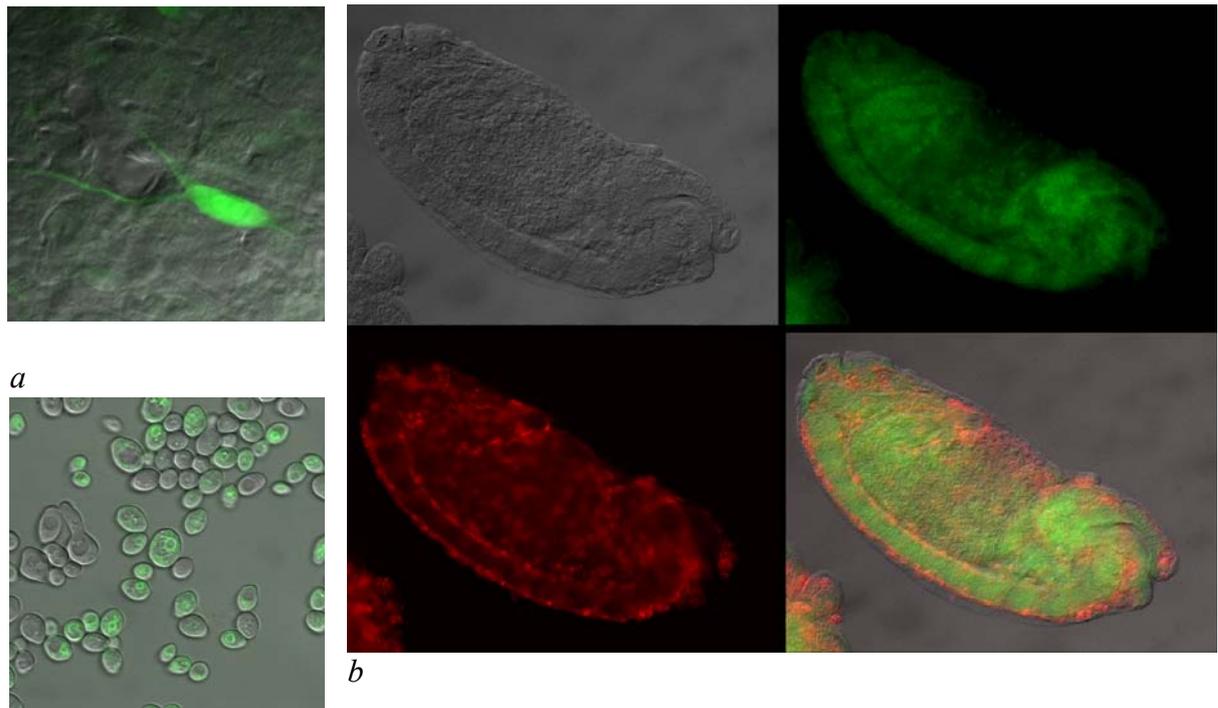


Fig. 19: Time lapse series of single cell electroporation of a pyramidal neuron with Ca^{2+} indicator Oregon Green Bapta-1: Overlay of fluorescence channel (non descanned detector) and transmission channel upon simultaneous acquisition by IR-light. Images taken in intervals of 280 ms. In the first frame the schematic drawing illustrates the experimental setup: A single pulse of -15V over 10 ms is delivered. (Nevian and Helmchen, 2007).

The advantage of the scanning gradient contrast is obvious: It is easy to view simultaneously the cell structure and the loading process of the fluorescence dye.

The Dodt gradient contrast works in camera- and scanning mode when using IR-excitation (see examples in Fig. 20). It can also be used upon VIS-excitation, but the contrasting effect can be less prominent.



c
 Fig. 20: Overlay of Dodt gradient contrast and fluorescence images: a) Mouse brain slice, Courtesy of Thomas Kuner, Inst. of Anatomy and Cell Biology, Heidelberg, Germany, b) *Drosophila* embryo: transmitted light, two fluorescence channels and overlay, c) Yeast cells: Courtesy of Prof. Sepp Kohlwein, ZMB - Zentrum für Molekulare Biowissenschaften, Karl-Franzens-Universität Graz, Austria

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