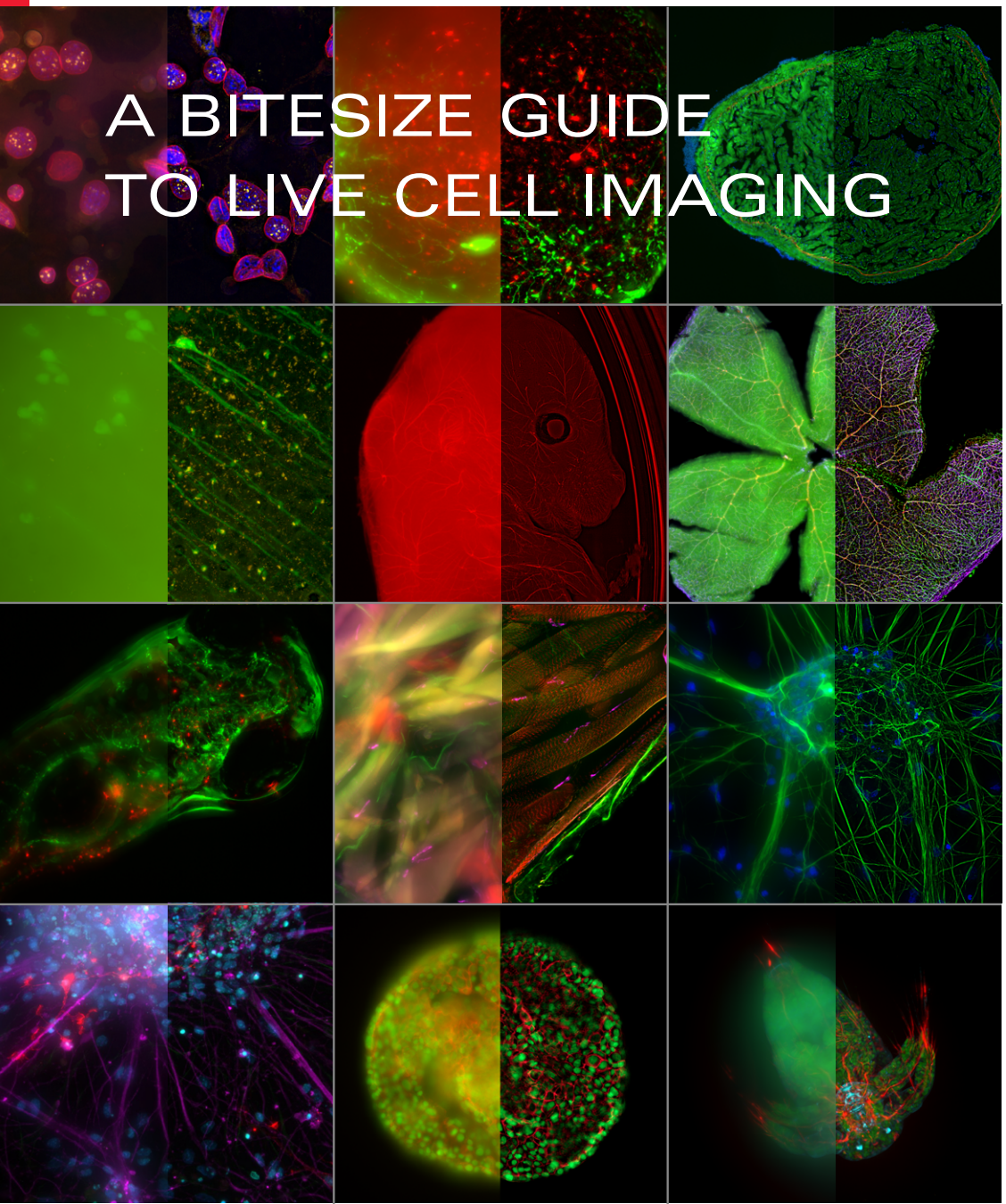


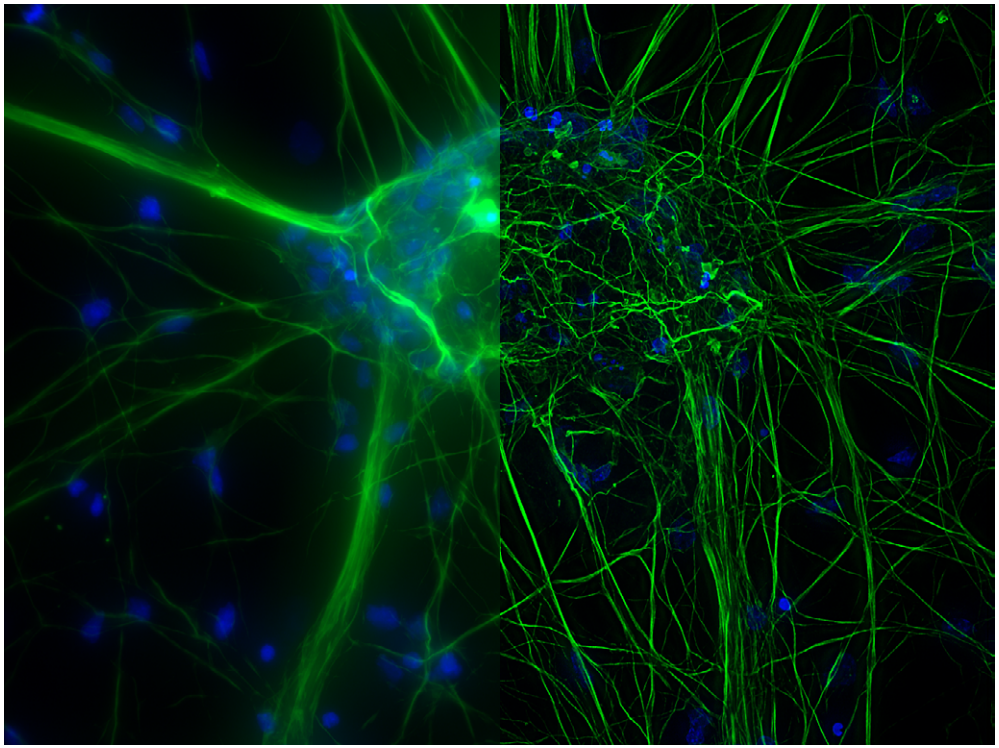
Page

An Introduction to Live Cell Imaging

The term live cell imaging refers to technologies used to capture images of cells in a living, active state. This includes static images, as well as time-lapse series. The use of live cell imaging has increased substantially over the years, as technological advances in electronics, data processing, optics, and fluorescent tag technology have made the technique more accessible, versatile, and affordable.

There are two main facets of live imaging that make it indispensable for modern research:

- The visual recording of cells in their living state – without any artefacts that could be potentially introduced by fixation or by the cessation of the various life processes itself.
- The capability to observe dynamic processes in cells, tissues, or whole organisms in real time, and in the native environment.



Cultured Cortical Neurons. Green, beta-III-tubulin; blue, Nuclei. Image stack of 59 planes for a volume of 21 µm, was acquired using a THUNDER Imager 3D Cell Culture. Raw image compared to one image acquired using THUNDER Large Volume Computational Clearing. Sample Courtesy: FAN GmbH, Magdeburg (Germany)

If physiological events on the organelle or molecular level are to be followed in real-time, then the sampling rate – frame rate, in this case – must be of a scale compatible with the speed with which these events progress. This can vary significantly and can be as short as milliseconds in some cases, for example in calcium imaging or the visualization of changes in the mitochondrial network.

During any imaging experiments, equal importance should be given to the health and stability of cells if the data to be collected from the observations are to be of real value. This means optimizing cell media, cell culture or observation vessels, sterile techniques, and avoiding phototoxicity as much as possible. As this is a key part of successful live cell imaging, the next part of our introduction deals with the various parameters that need to be considered to maintain cell viability.

Preservation of ...

Cell Media

Mammalian cells must be kept at a constant temperature of 37 °C. Moreover, as cells metabolize, their metabolic products lower the pH of the culture medium. To counteract this acidification and maintain a pH 7.4, a buffer system of bicarbonate (HCO_3^-) and dissolved carbon dioxide (CO_2) is used, the latter of which originates as 5% atmospheric CO_2 from cell culture incubators. When no CO_2 is available, then a buffer containing 10 to 20 mM HEPES (2-[4-(2-hydroxyethyl) piperazin-1-yl] ethanesulfonic acid), a zwitterionic organic chemical buffer, can also be used. Phenol red in the medium is a usual method of monitoring pH levels and its color gradually changes from yellow to red over the pH range 6.8 to 8.2. Above pH 8.2 it becomes pink. For fluorescence imaging, cells should be placed in phenol red-free medium as it can give a strong fluorescent background that decreases the contrast.

Appropriate media content is also of paramount importance for cell survival in vitro. Major constituents of the cell media include glucose, sodium pyruvate, amino acids, vitamins, and inorganic salts. Fetal Bovine Serum (FBS) is often used as a source of proteins, lipids, and growth factors, when a defined medium is not required.

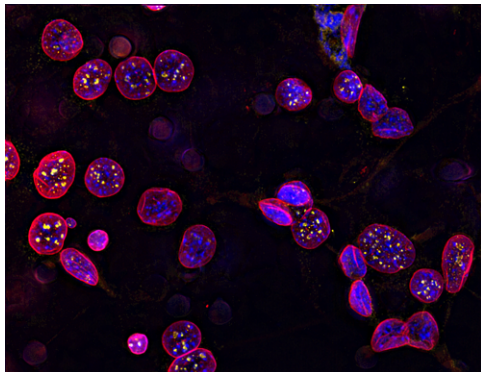
Cell culture vessels

Cell culture vessels can vary in size, from 96-well plates to large scale containers. Plastic-bottomed containers can be used for low magnifications, but for high magnification immersion objectives, glass bottomed containers, with specific glass thickness and refractive index, must be used. Imaging cells in routine culture vessels can result in mismatched refractive indices that may lead to image degradation due to reduced resolution, aberrations and contrast. In addition, some materials used to manufacture culture vessels may autofluoresce at certain excitation wavelengths. The consideration of culture vessel needs to be balanced against the cells' preferential growth on plastic, and the need for coating with substrates such as poly-l-lysine or poly-l-ornithine when using glass.

A number of manufacturers offer cell culture vessels that provide solutions relating to the healthy growth of cells that also take into account imaging parameters. These include adhesive culture vessels that can be stuck to a preferential base substrate, or vessels manufactured from polymers that have a specific refractive index.

Sterile Technique

Sterility is always a prerequisite for cell culture applications, regardless of whether they are used for microscopy or not. Bacterial contamination is an ever-present concern; therefore antibiotics are very frequently added to cell culture media (1). The most common antibiotic scheme for cell culture is a combination of penicillin, that impairs bacterial wall synthesis, and streptomycin, that interferes with protein biosynthesis and causes cell death. Sometimes a specific protocol requires that antibiotics are avoided, for example in some embryonic stem cell cultures (2) – in which case meticulousness in handling and maintaining sterile conditions becomes even more important.



C2C12 cells stained with lamin B (magenta) for nuclear structure, Hoechst (blue) for DNA, and H2AX (yellow) for DNA damage. Cells were imaged using a THUNDER Live Cell Culture imaging system with a 63X/1.4 oil immersion objective. Images above represent extended depth of field projections of 17.47 mm thick z-stacks. Images courtesy of Dr. Lucas Smith, Department of Neurobiology, Physiology and Behavior, College of Biological Sciences, University of California at Davis, Davis CA.

Important Considerations in Live Cell Microscopy

If cells are to be used for live imaging, then a number of conditions have to be satisfied to ensure that both the health of the cells is maintained, and the appropriate imaging conditions are met.

Regulation or pH

For some cell types, pH regulation can be achieved through the use of HEPES or other chemical buffers (e.g., MOPS, TES), as already mentioned. But in other cases, a pH-buffering bicarbonate in the medium is necessary. This can be addressed in two ways. One is by constantly delivering gaseous carbon dioxide (usually in the form of carbogen: 95% oxygen with 5% carbon dioxide) to the medium and constantly superfusing the cells. This method is most commonly used for acute preparations with very high metabolic turnover, for example brain slice preparations. Another common approach, mainly used with long-term cell culture experiments, is to surround the cells with a culture chamber that is designed to maintain a regulated atmosphere of 5% to 7% CO_2, regulated temperature (usually 37 °C), and humidity (95%). Usually a large, temperature-regulated chamber completely surrounds the microscope stage, objectives, fluorescence filters, and transmitted light condenser, as well as the specimen and a second, smaller chamber surrounds the sample, providing appropriate humidity and gas content.

An alternative solution is a small chamber (sometimes called a "water jacket stage incubator") that provides control for all three parameters – temperature, humidity and gas.

Choice of Microscope

An inverted microscope is routinely used for live cell imaging applications. Using this configuration provides a number of advantages over a standard upright microscope:

- **Working distance of objectives.** This is defined as the distance between an objective front lens and the surface of a specimen when that specimen is in sharp focus. Imaging cells in a culture vessel using an upright microscope configuration would require an objective to focus through the material of the vessel, an air gap, and the cellular medium. Such a physical distance is out of the working distance of many objectives, especially higher magnification objectives that have working distances less than one millimetre.
  In contrast, the configuration of an inverted microscope significantly reduces the distance between the objective front lens and the specimen allowing sharp focus to be achieved through the base plate of a culture vessel.

- **Sample access to the cell media.** As the space above a culture vessel on an inverted microscope stage is freely accessible, this allows samples to be taken from the media, or substances can be added to the cells during imaging experiments.

- **Optimal focusing of more cells.** Suspended cells generally settle to the bottom of a culture vessel, and can therefore be observed on the same focal plane. Although water dipping objectives can be used to image cells on an upright microscope, this can increase the risk of contamination as the objective front lens is dipped into cellular media during imaging. Water immersion objectives can be used in an inverted microscope configuration with water replacing the air gap between the objective front lens and the culture vessel to closely match the refractive index of the aqueous medium.

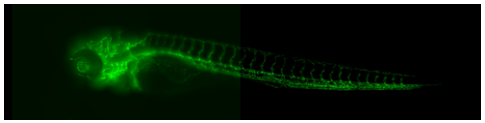
Phototoxicity

Fluorescence imaging can interfere with cellular homeostasis due to phototoxicity. Phototoxicity can originate from a number of sources. Organic molecules present in the cells, such as flavins and porphyrins, absorb light and become degraded when they react with oxygen. This reaction leads to the production of reactive oxygen species (ROS) including superoxide radicals, hydroxyl radicals, and hydrogen peroxide that can damage cells. Phototoxicity also occurs when synthetic fluorescent dyes are excited by laser irradiation or with a high intensity arc-discharge lamp. In their excited state many of these dyes react with molecular oxygen and produce toxic free radicals (3).

The DMi1 from Leica Microsystems, shows the basic setup and design of an inverted microscope specifically designed for cell and tissue culture, with a petri dish containing live cells on the microscope's stage.

There are a number of steps that can be taken to reduce phototoxicity:

- Using fluorophores that are excited by lower energy (longer wavelength) light.
- Using the lowest possible light intensity.
- Using the shortest possible excitation duration.
- Reducing the frame rate as much as possible, especially in long experiments.
- Improving the intensity and signal-to-noise ratio by using camera features such as binning or gain, or by using special low light cameras (e.g., electron-multiplying charge-coupled device (EM-CCD) cameras or scientific CMOS (sCMOS) cameras), as opposed to increasing excitation. A compromise should be reached between optimum conditions for cell health and optimum conditions for imaging. Sub-optimal imaging conditions may lead to inaccuracies or inconsistent data, whereas over-exposure of cells to light can result in phototoxicity.
- Remembering that multiple rounds of low irradiation are less harmful than a single instance of the same total energy. This is especially relevant for confocal microscopy, where it is preferable to use low excitation, high gain and higher averaging, to achieve the same results that would be achieved by stronger excitation, lower gain and little or no averaging.



Zebrafish larvae (72 hours post fertilization). Blood vessels (green). Sample courtesy: Dr. Almary Guerra & Dr. Didier Stainier, Max Planck Institute for Heart and Lung Research, Bad Nauheim (Germany).

Focus Drift

Focus drift is another factor to be taken into consideration in live cell imaging, especially in long term experiments. Focus drift can result from temperature changes, coverslip flex due to thermal gradients and other variations in culture chamber temperature including imaging chamber temperature in-homogeneities. Other contributing factors include mechanical instability of the focusing mechanism, addition of media or reagents and, of course, specimen movement in live cell imaging.

Some of these problems can be controlled by appropriate design of the microscope, incubation chambers, anti-vibration microscope tables and thermal stability in imaging rooms through the use of high precision air conditioning systems. Any occurring drift can be compensated through hardware-based autofocus systems, while the problems created by live cell movement can be addressed by using software (image)-based autofocus systems.

As a general rule, at least 2% contrast is required for the human visual system to differentiate a detail in an object or an object from its background. However, live cells are essentially colorless, and it is sometimes the case that the experimental setup requires that no contrasting dyes or labels are used.

In these instances, microscopy techniques take advantage of light phenomena such as reflectance, birefringence, light scattering, and diffraction to achieve optimum contrast without employing any dyes.

Phase Contrast Microscopy

The phase contrast technique was developed in the 1930s by Frits Zernike, and first used in 1942. Zernike was awarded the Nobel Prize in Physics in 1953 for his invention (4). Phase contrast works by translating otherwise invisible phase variations, which occur upon interaction of light with biological structures, into corresponding amplitude variations, creating image contrast (Figure 1). This technique is ideal for thin specimens such as cells on glass, which are approximately 5 to 10 micrometers thick in the center, above the nucleus, and less than a micrometer thick at the periphery.

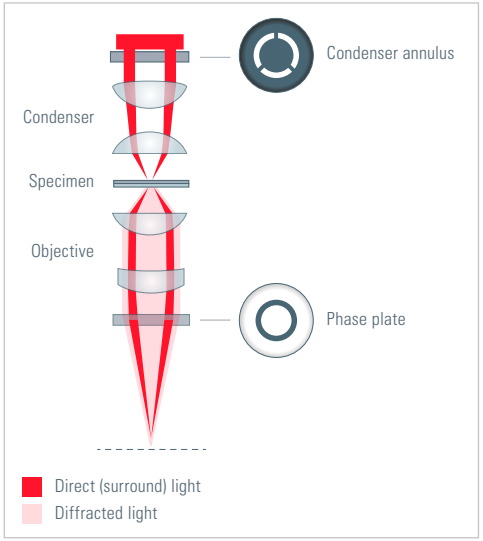
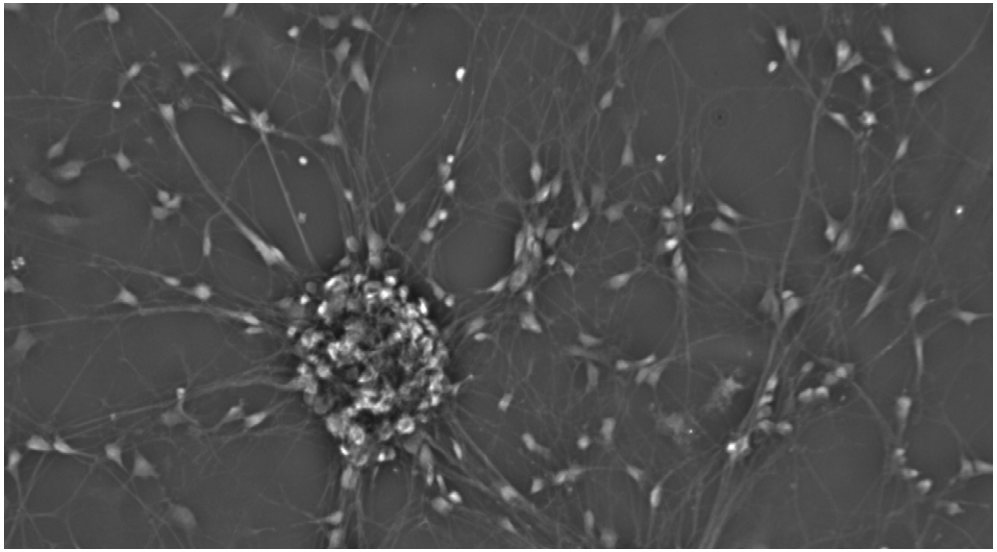


Figure 1: Optical path in a phase contrast microscope. Ring shaped light that passed the condenser annulus is focused on the specimen by the condenser. Portions of the ring shaped light are diffracted by optically dense structures of the specimen (e.g., plasma membranes, organelles etc.) and experiences a phase shift of approximately ¼ λ (usual for biological specimen). This phase shifted and diffracted light bypasses the phase ring and is hardly affected by the phase ring (mostly located in the rear focal plane of the objective). In contrast, the direct ring shaped light from the condenser annulus will hit the phase ring, which will dim the direct light and cause a phase shift (usually advanced by ¼ λ or retarded by ¾ λ for positive phase contrast). As the total phase shift between the light refracted by the specimen and the light that passed the phase ring will be up to ½ λ, destructive interference will occur. Optically dense structures will consequently appear darker (in positive phase contrast).

This technique is an excellent way to increase contrast in live, unstained cells. However, phase contrast microscopy can result in artefacts in the form of bright halos around the specimens or other areas of contrast, created by inadvertent transmission of some of the diffracted light through the circular phase-advancing neutral density ring in the objective phase plate. Phase contrast microscopy is not appropriate for thick specimens, as their overlapping structures produce more severe halo artefacts.



An image of neurons captured using phase contrast.

Differential Interference Contrast (DIC) Microscopy

DIC microscopy is an imaging technique that produces images of fine structures in cells using polarized light to detect differences in the refractory index. It can be retrofitted to many microscopes, with the addition of the appropriate components (Figure 2).

DIC microscopy provides high resolution images of unstained biological samples, producing relief-like images in which objects appear to have a shadow cast. There are no halo artefacts and relatively thick specimens can be imaged. However, DIC microscopy is only suitable for glass-bottomed culture plates, as plastic does not preserve the polarization of light, and thus results in loss of contrast.

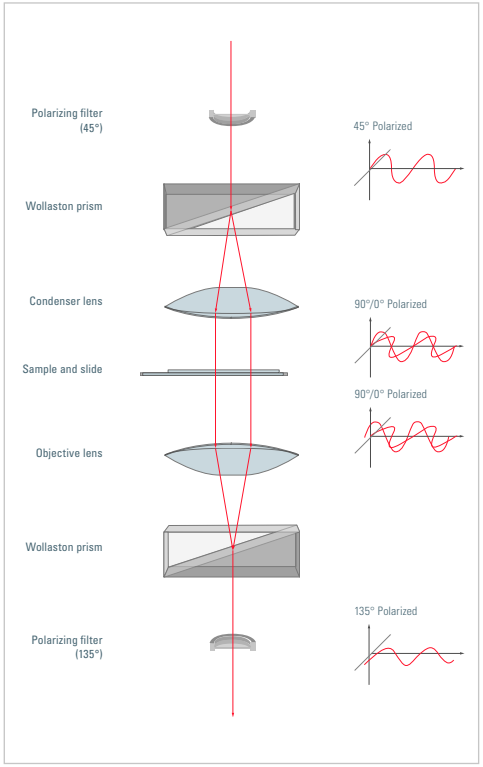


Figure 2: Unpolarized light from a light source passes through a polarizing filter and is polarized at 45°. After passing through the Wollaston prism, the light is separated into perpendicular polarized components, one with 0° and one with 90° polarization. The condenser subsequently directs the light through the sample. The sample is illuminated by two coherent parallel light rays with different polarization. This basically produces two slightly offset brightfield images of the sample, one with 0° and one with 90° polarized light. Due to the different polarization of the light, these images do not interfere. The separated rays experience different optical path lengths, as there might be differences in the thickness or refractive index at the point they pass through the sample. This results in a phase shift of one ray compared to the other. After passing through the objective, the perpendicularly polarized light is recombined to one polarized at 135°. According to the differences in the optical path length, interference of the two rays now produces brightening or darkening, making otherwise hardly visible structures appear. Finally, directly transmitted light, which did not experience any phase shift, is removed by the polarizing filter with 135° direction (also called an "analyser").

Integrated Modulation Contrast or Hoffman Modulation Contrast (IMC/HMC)

IMC microscopy achieves enhanced contrast in live specimens by converting phase gradients in the sample into amplitude (brightness) differences and creates pseudo-3D images of unstained samples (Figure 3).

It employs a Hoffman modulator, which is a zoned optical amplitude filter, inserted in the back focal plane of the objective. This filter has three zones:(1) a small, dark zone transmitting only 1% of light; (2) a narrow gray zone transmitting 15%; and (3) a transparent zone, transmitting 100% of the light. A slit aperture is placed off-axis in the condenser front focal plane, creating oblique illumination through the specimen. The slit is adjusted so that light transmission falls on the gray region (15%) of the modulator. Non-refracted light transmitted through the specimen consequently passes through the gray region in the center of the modulator and produces a subsequent gray image. However, where the sample structure introduces refraction, the light path will be shifted toward either the transparent zone or the near-opaque zone, introducing contrast and creating pseudo-3D images.

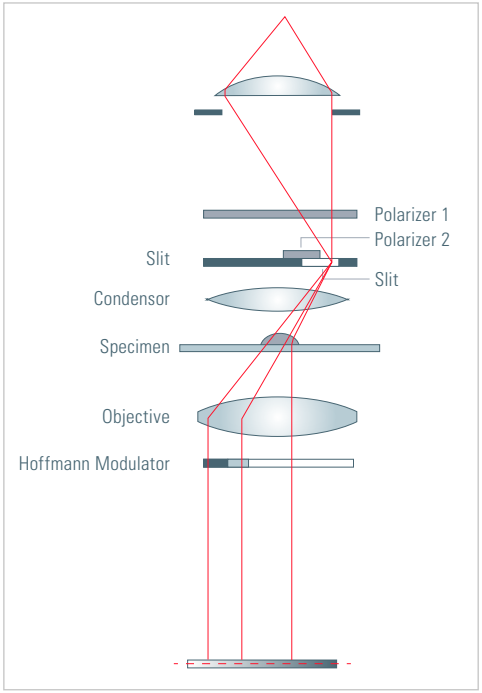
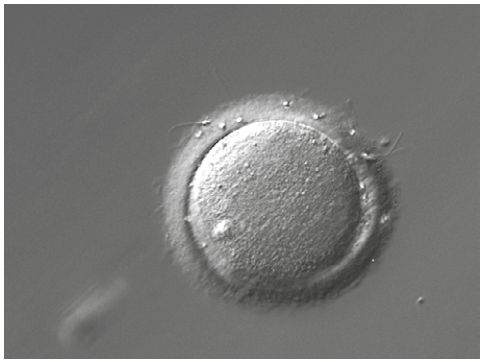


Figure 3: Light from the condenser passes the slit of the slit plate to enable oblique illumination. When the light passes the specimen, it is deflected at phase transitions (e.g. changing slope of the plasma membrane). Subsequently the light is deflected to the opaque or transparent parts of the modulator producing a relief-like, three-dimensional image impression.

Unlike DIC microscopy, there are no beam-splitting prisms in IMC, and the two polarizing filters ("Polarizer 1" and "Polarizer 2" in Figure 3) are placed optically in front of the sample, meaning that the technique can be used with plastic culture vessels. For Leica Modulation Contrast, no special objectives are needed, and objectives up to 63x with high correction (e.g., planachromatic and apochromatic objectives) can be used. IMC microscopy produces images with good contrast and high resolution.

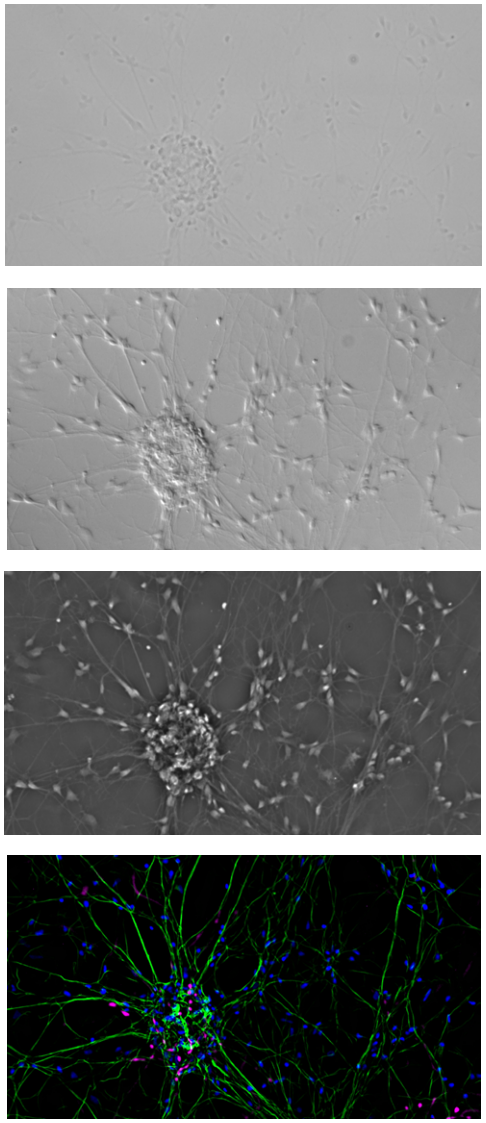


Hard,contrast-pronounced modulation contrast. C. Mehnert, Center for In-Vitro Fertilization, Gießen, Germany.

Fluorescence Microscopy

Fluorescence is a form of luminescence and encompasses a large number of techniques that collectively make up a considerable proportion of all microscopy experiments. Fluorescence microscopy utilizes the properties of fluorophores (inorganic dyes, proteins, etc.) that emit a longer wavelength of light upon excitation by light of a shorter wavelength. Fluorophores are categorized by their excitation and emission peaks, and the difference in these peak wavelengths is known as the 'Stokes Shift'.

From the construction of the first fluorescence microscope in 1911 through to the discovery of Green Fluorescent Protein in 1962, fluorescence microscopy has continually evolved and advanced, and is one of the most essential components of modern microscopy's toolbox. Fluorescence microscopy can be used to determine the distribution, quantification and localization of a single molecule species in the cell. Colocalization and interaction studies can also be performed, ion concentrations can be estimated and cellular processes such as endocytosis and exocytosis can be observed.



Fluorophores can be used in a number of ways, for example they can be attached to antibodies in single- or multi-step immunofluorescence experiments, expressed in genetically engineered cells and whole organisms, or as sensors added to cell cultures to investigate biochemical changes in the cells by examining their fluorescence properties. Advances in the field of fluorescence microscopy have included the introduction of quantitative methods including the so-called "F-techniques" (FLIM, FRET, FRAP, etc.) as well as photomanipulation techniques and optogenetics (see below).

Left: Neurons imaged from top to bottom in brightfield, phase contrast, DIC and fluorescence.

Total Internal Reflection Fluorescence (TIRF) Microscopy

TIRF microscopy is a special technique used for observing processes that are in, or close to, the plasma membrane of a cell. Instead of direct illumination via light delivered by an arc lamp, LEDs, or lasers, TIRF microscopy uses an evanescent field for fluorophore excitation that reaches a depth of between 60 to 250 nm from the coverslip, providing excellent Z-resolution and enabling the imaging of events such as molecular transport to the plasma membrane. The evanescent field occurs if incident light is totally reflected at the interface of two transparent media with different refractive indices (Figure 4).

In biological applications, the incident light is usually produced by a laser, and the interfaces that provide the requirements for TIRF can occur between a coverslip and a film of aqueous solution between the coverslip and adherent cells, or between the glass and the cell membrane itself, if no medium is present. As TIRF depends on reflection at the interface, any irregularities present in the glass slide or coverslip would be detrimental. Hence, high precision glass coverslips are required as they play an instrumental role in this imaging technique. As the energy of an evanescent field decreases exponentially with increasing distance from the interface, only

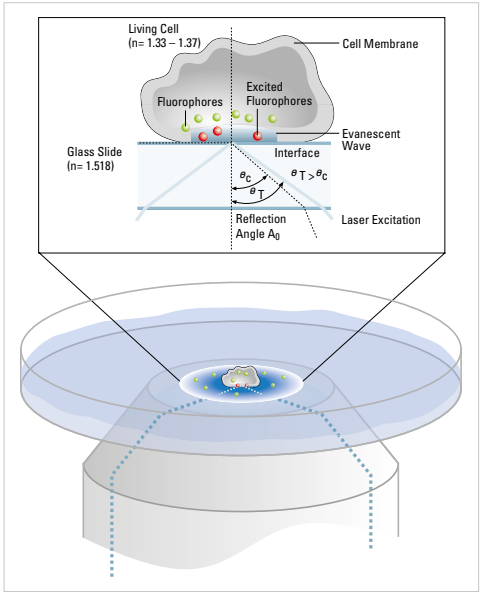


Figure 4: The principle of TIRF microscopy.

fluorophores in close proximity to the coverslip are excited, resulting in an optimal signal-to-noise ratio. TIRF microscopy also has low phototoxicity and allows for a high image acquisition speed.

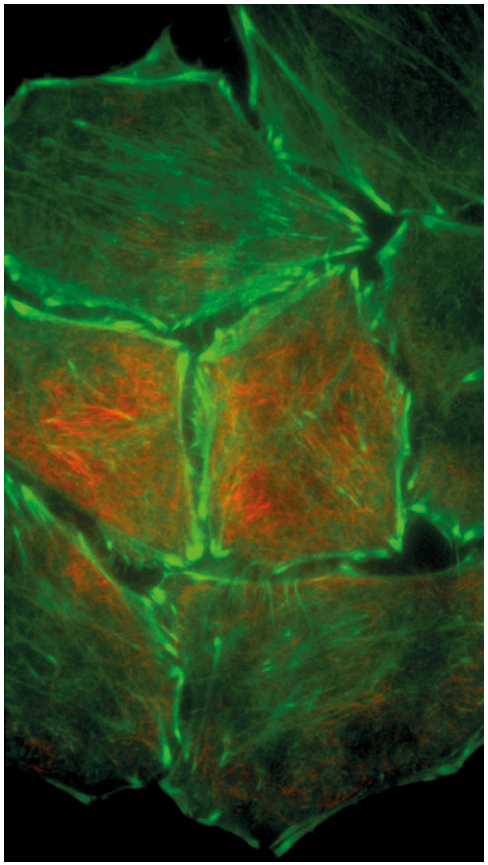


Figure 5: Hela cells stably expressing Actin Chromobody-TagGFP2 and stained with SIR-Tubulin. Courtesy of ChromoTek GmbH, Munich, Germany, and Spirochrome SA.

Ion Imaging

Ion imaging is used to observe changes in ion concentrations (i.e., calcium, chloride, magnesium etc.) in cells and the extracellular space. Ion regulation in both spatial and temporal terms is of major interest for life sciences research, as many fundamental functions of a cell strongly depend on delicate but dynamic balances of ions, voltage potentials, and pH. For example, the excitability of neurons, muscle contraction, cell movement, gene transcription, and other processes are determined by the ion concentration of the cytosol or specific intracellular compartments.

Ion imaging is performed using either fluorescent dyes or proteins especially designed to respond to ion binding by changing their emission profile. For example, Fluo-4 increases its fluorescence emission over 100-fold upon binding to calcium ions. For sodium detection, SBFI (sodium-binding benzofurzanisophthalate) or Sodium Green™ are commonly used, while PBFI (potassium-binding benzofurzanisophthalate) detects potassium ions.

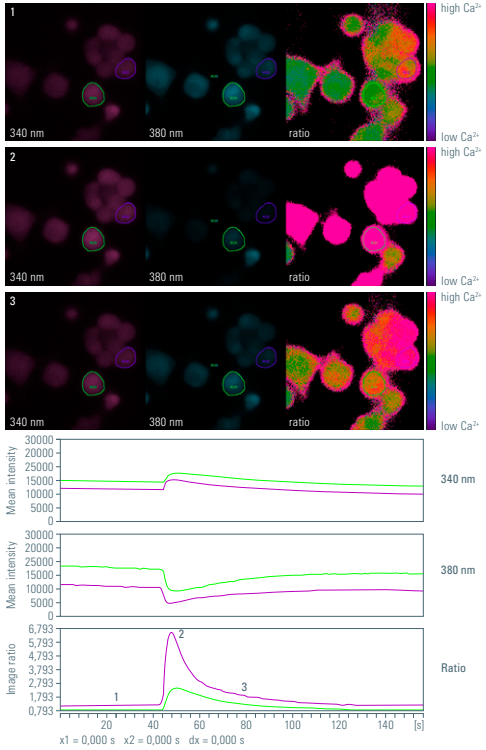


Figure 6: Snapshots from a time lapse of a calcium imaging experiment using the ratiometric calcium indicator Fura-2. Depicted are false-color images after 340 nm (left) and 380 nm (middle) excitation and the corresponding calculated ratio image (right). The image series shows three points in time: At the first point in time (1) cells are not stimulated and the intracellular calcium is at resting level. At the second point in time (2) the cells are stimulated and the calcium is at maximum level. At the third point in time (3) the intracellular calcium level is declining. In the graph the corresponding points in time are marked. The upper graph shows the intensity of the 340 nm image, the middle graph that of the 380 nm images and the lower graph shows the ratio intensities.

The precise estimation of ion concentrations, or relative changes in locality in a cell or a cellular network, is often difficult to achieve using conventional fluorescence methods. This is due to changes in cellular morphology / ion distribution that can influence the emitted light, leading to substantial misinterpretations when dynamic changes of ion concentrations, voltage or pH are investigated. Ratiometric imaging techniques bypass these issues by observing emission or excitation wavelength shifts of fluorophores, or by comparing the emission intensity of a fluorophore combination instead of measuring intensity changes.

For example, in the "classic" Fura-2 calcium dye, the 340 / 380 nm excitation ratio allows accurate measurements of intracellular Ca^{2+} concentration (see Figure 6 for an example of a calcium imaging experiment using Fura-2).

Fluorescent Lifetime Imaging Microscopy (FLIM)

Fluorescent Lifetime Imaging Microscopy (FLIM) allows researchers to study cellular processes by analyzing the fluorescence lifetime properties of a fluorescent dye. In time-domain FLIM, fluorescent molecules are excited using pulsed illumination with pulses of a very high frequency (40 to 80 MHz), and the emitted photons can be registered and analyzed during the short exponential decay period of several nanoseconds after each pulse. Fluorescence lifetime is defined as the average time that a molecule remains in an excited state prior to returning to the ground state by emitting a photon, and can give valuable information on biochemical changes in extra- and intracellular environment, as they are reflected in changes of the lifetime of a fluorophore (information that cannot be derived from intensity measurements alone). One can use FLIM to follow fast molecular interactions via FLIM-FRET (Förster Resonance Energy Transfer), whereby the energy transfer is detected by donor lifetime changes (see below), use biosensors to detect changes in

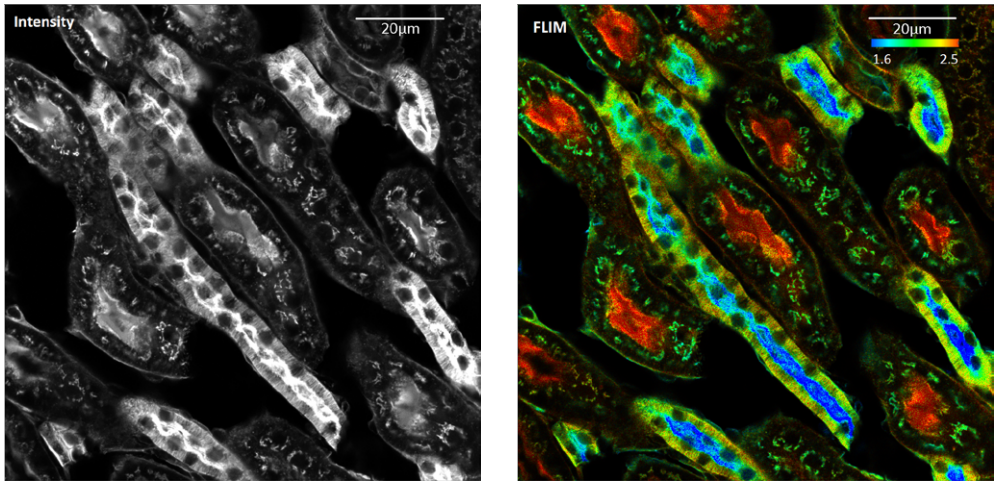


Figure 7: Kidney slice (FluoCells® prepared slide #3). The cell membranes are stained with WGA-Alexa Fluor488. The lifetime contrast displayed in rainbow scale indicates that the membranes are located in very different micro-environmental conditions, e.g. different pH or ion-concentrations. Color bar scale (lifetime): nanoseconds.

metabolic state and microenvironment, or apply lifetime contrast to separate multiple fluorophores of similar emission spectra. This method is advantageous since the data is not dependent on the intensity of the signal, and is therefore not influenced by artefacts such as photobleaching and concentration variation. FLIM requires pulsed laser sources and single-photon counters with low noise and high precision to detect photon arrival times, combined with timing electronics capable of processing the signals generated by the detectors without impairing their performance.

Figure 7 shows an example application of FLIM used to examine the different microenvironments in a section of kidney.

Photomanipulation Techniques

The term "photomanipulation" describes a wide range of techniques that enable the investigator to interact with the observed phenomena in a specimen through the use of light. The following techniques are also live cell imaging techniques, but with the added component of intervention, which allows access to information that would not be available from observation alone.

> **FRET, FLIM FRET and BRET – Quantifying Protein-Protein Interactions**

FRET and BRET (bioluminescence resonance energy transfer) are techniques whereby energy is transferred between fluorophores (Figure 8). FRET can be used for the quantification of dynamic processes inside the cell, such as protein conformational changes, protein-protein interactions, and protein-DNA interactions.

Proteins of the GFP family, commonly CFP / YFP, FITC / TRITC, Cy3 / Cy5, EGFP / Cy3 and many others, are attached to the proteins of interest using molecular biology methods. The donor is excited using the appropriate wave length of light and, if the distance between donor and acceptor is very close (usually in the range between 1 and 10 nm, often around 5 nm), then the donor will transfer energy to the acceptor. The result will be a shift from blue fluorescence emitted from the CFP to yellow fluorescence emitted from the YFP, in the case of this particular pair, or the corresponding changes in other pairs.

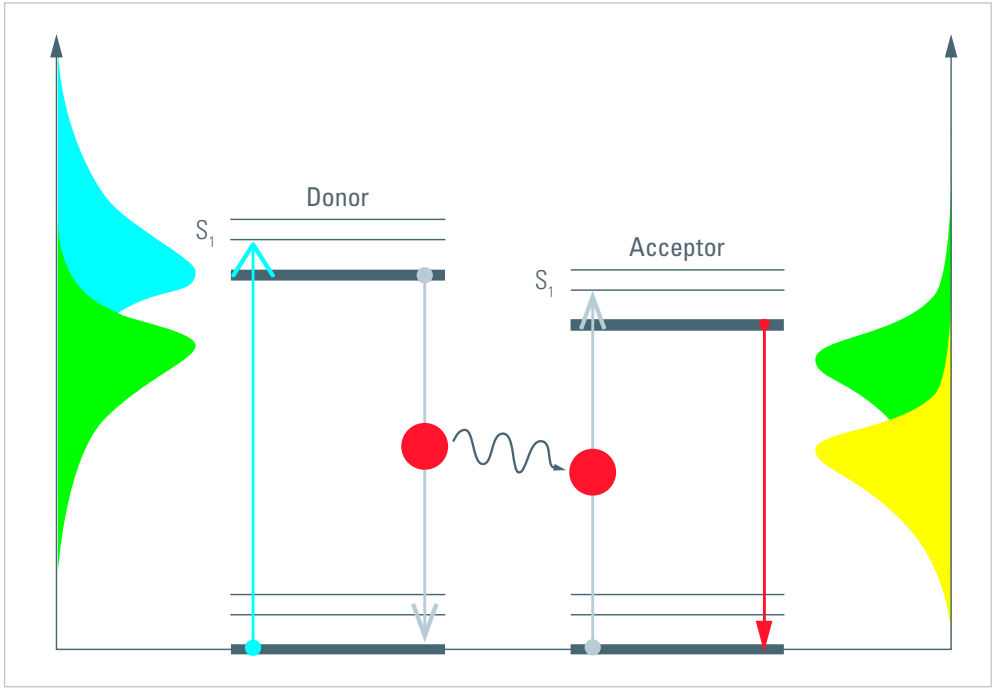


Figure 8: Energy transitions in a FRET pair. Light energy matching a transition in the donor molecule is absorbed (blue arrow). The excited donor can relax either by fluorescence (gray dotted arrow, left) or by resonance energy transfer to the acceptor molecule (black arrow).

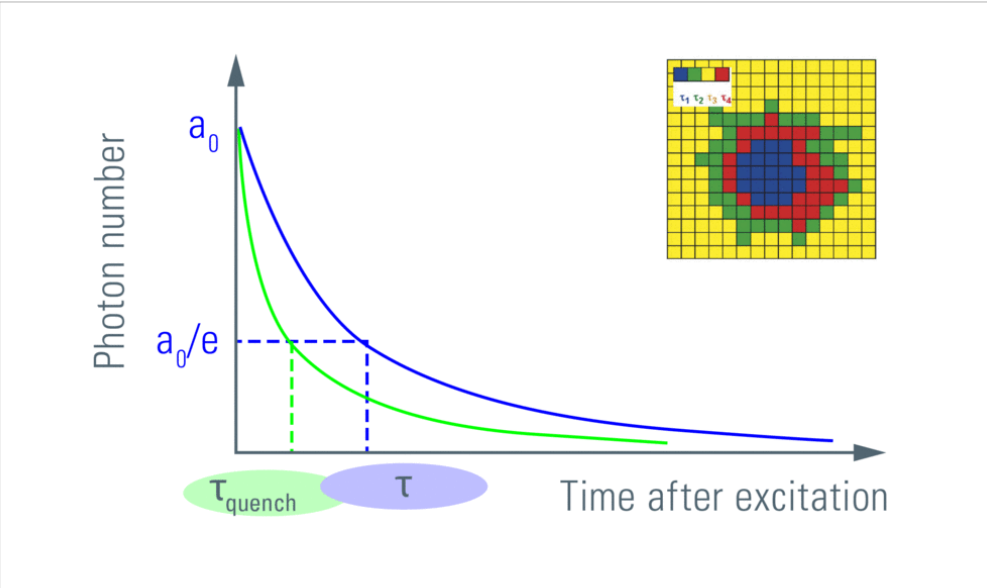
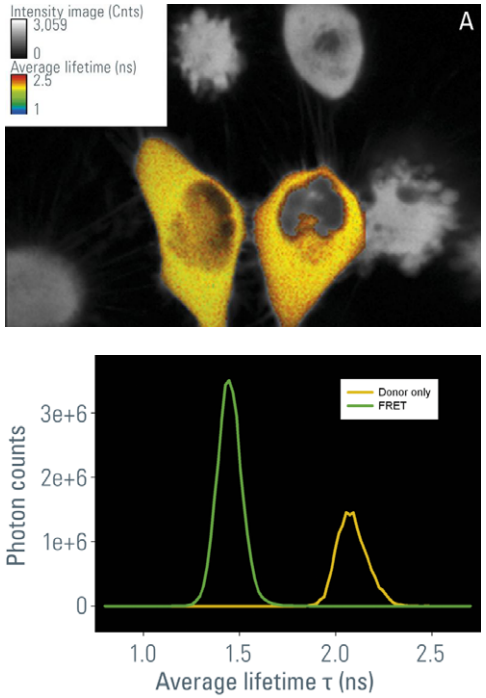


Figure 9: Plotting the fluorescence photon number over elapsed time after excitation. The initial number of emitted photons after the excitation pulse, a_0, decays exponentially. The fluorescence takes time to decay to a_0/e (~37%) is the fluorescence lifetime. Lifetime τ shifts to shorter times due to FRET (T_{quench}). Another read-out from the lifetime decay is the amplitude a_0. Measuring the lifetime at each position in a scanning system yields a spatial map of lifetimes (see inset).



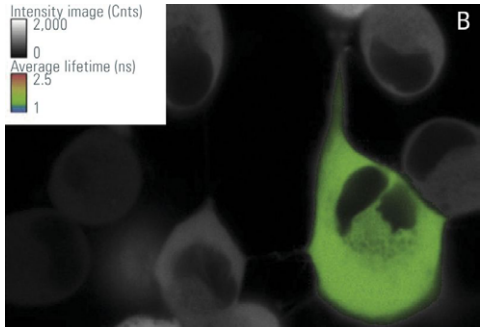


Figure 10: Top row: RBKB78 cells transfected with a CFP donor only (A) and CFP-YFP fusion (B). The detection band was set between 445–495 nm using spectral FLIM detectors. The colored region has been used for analysis. Colors represent intensity modulated fluorescence lifetimes. Courtesy of Prof. Gregory Harms, University of Würzburg, Germany. We acknowledge experimental support by Dr. Benedikt Krämer (Picoquant, Berlin), Jan-Hendrik Spille and Wiebke Buck. Bottom row: Fluorescence lifetime distribution of donor only (yellow) and FRET (green) samples using average lifetimes. There is a clear shift of 0.7 ns towards shorter lifetimes in the FRET sample.

The lifetime of the fluorescence emitted by CFP will also change, due to energy absorption by the acceptor protein (Figure 9); therefore an alternative way to detect the energy transfer is by detecting this change using FLIM (see above). This method is termed "FLIM-FRET".

The reduction of the fluorescence lifetime is a kinetic parameter that can be determined independently of brightness, probe concentration, or the existence of moderate levels of photobleaching, making FLIM-FRET a very accurate quantitative estimate of FRET efficiency (Figure 10 illustrates an example of FLIM-FRET in live cells).

In the case of BRET, the donor is a bioluminescent molecule (e.g., luciferase derivatives) acting as the donor while a molecule of the GFP family acts as the acceptor.

> FRAP, iFRAP and FLIP

Three main microscopy techniques are used to monitor protein and vesicle trafficking: FRAP, iFRAP, and FLIP (Figure 11).

The FRAP (Fluorescence recovery after photobleaching) technique uses a fusion protein consisting of a fluorescent protein (usually GFP or one of its derivatives) and a protein of interest whose movement is to be monitored. Often, this protein is distributed widely inside the cell, so that the entire cell appears fluorescent. The investigator exposes a section of the cell to high intensity excitation light, usually from a laser source, until that section of the cell is bleached. During the "recovery" period following photobleaching, proteins, including the tagged protein of interest, will gradually diffuse back into the bleached area, restoring fluorescence. The rate of fluorescence recovery provides the investigator with information on intracellular transport dynamics. During inverse FRAP (iFRAP), a portion of a cell outside the region of interest is photobleached, the rationale being the same as in FRAP.

Unlike FRAP, which is used to examine the kinetics of recovery following one photobleaching event, FLIP (Fluorescence loss in photobleaching) is used to understand how the loss of fluorescence can permeate an entire cell after several photobleaching events. In FLIP, a selected area just outside the region of interest is continuously photobleached. The rate of bleaching of the rest of the cell is used to measure the trafficking of a tagged protein throughout the whole cell, and helps identify regions of higher and lower turnover in different cellular compartments.

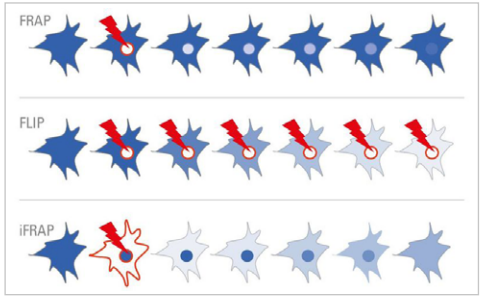


Figure 11: During fluorescence recovery after photobleaching (FRAP) the fluorescence tag of a protein is bleached by powerful excitation inside a region of interest (ROI). Subsequent resettlement gives insight e.g. into the turnover of the structure of interest. For fluorescence loss in photobleaching (FLIP) a ROI within a cell is bleached repeatedly. The bleached molecules spread out and the loss of fluorescence indicates, e.g. whether cell organelles are physically connected. During inverse FRAP (iFRAP) the cell region outside the ROI is bleached. Subsequently the non-bleached molecules spread out and can be monitored.

> Photoactivation, Photoconversion and Photoswitching

Photomanipulation can be used to activate or change the emission response of photosensitive fluorescent proteins. More specifically, fluorophores can be "switched on" from a low fluorescent state to a higher fluorescent state in less than a second with a short light pulse in the violet / blue part of the spectrum; this is termed "photoactivation". "Photoconversion" occurs when an already fluorescent protein changes its excitation and / or emission spectrum after exposure to a short pulse of light.

While photoactivation is an irreversible conversion from a non-fluorescent state to a fluorescent state, photoswitchable proteins are able to shuttle between the two conditions, using light pulses of different wavelengths. This can be performed several hundred times without photo-bleaching (Figure 12).

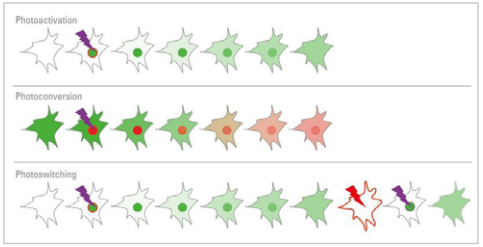


Figure 12: Photoactivation, photoconversion, and photoswitching can be used to track protein populations over time. Photoactivatable proteins can be "switched on" by light of a certain wavelength. Photoconvertible proteins can change their emission spectrum, whereas photoswitchable proteins can be switched on and off many times.

Photochromic fluorophores can switch between fluorescent and dark states and are especially useful when investigating the behavior of abundantly expressed proteins. In many cases, the proteins used are genetically fused to proteins of interest whose expression or transport can then be monitored. Methods like FRAP or particle tracking can then be applied to further investigate the protein of interest.

> Optogenetics

Optogenetics enables investigators to selectively activate biological pathways using photo sensitive protein domains. Commonly, optogenetics involves targeted activation of photo sensitive ion channels to change the membrane voltage potential (Figure 13).

The effect of the activation can be measured using electrophysiology or through optical sensors such as ratiometric calcium dyes. Photo sensitive ion channels allow the stimulation of neurons and other cell types with high spatial and temporal resolution. In addition to receptor-based optogenetic "switches", new optogenetics tools are emerging through the use of many other photo sensitive protein domains.

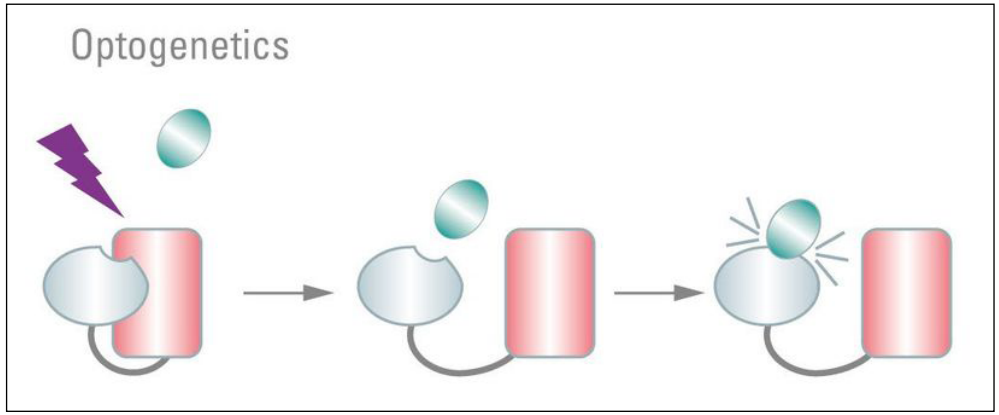


Figure 13: Optogenetics utilizes photo sensitive protein domains to trigger cellular mechanisms. Classically, photo sensitive ion channels were used to change the membrane potential. Meanwhile additional proteins have been domesticated which show conformational changes after irradiation with light. This can be used to activate or deactivate target proteins e.g. to induce protein resp. DNA binding or enzyme activation.

> Cutting and ablation

One form of photomanipulation is to use light to physically destroy parts of the specimen with high power pulsed lasers. Depending on the power applied to the sample, effects can range from cutting individual cytoskeletal fibers or amputations of parts of a cell, through to total ablation of cell clusters or organs.

These techniques find use in a wide range of applications, from probing the role of cytoskeletal elements, to investigating the mechanics of inflammation and wound healing, as well as neuronal regeneration (Figure 14).

Developmental biology is another field where such techniques find application, whereby investigators can ablate specific parts of an embryo and record the impact on its development.

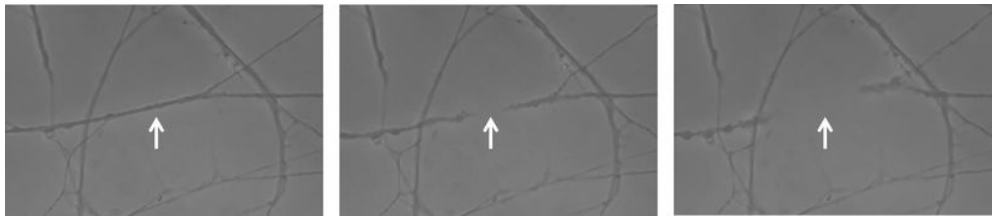


Figure 14: To investigate neuronal regeneration capabilities, axons can be cut with the help of a pulsed laser (arrow). Ablation and cutting experiments range from cutting whole cells or cell fragments down to single cytoskeletal elements. Image courtesy of Richard Eva, Cambridge University (UK).

> Uncaging

Chemically encapsulated compounds that are inert when intact can be designed to degrade to biologically active compounds following exposure to UV light (Figure 15).

Such compounds are referred to as “caged compounds” and targeted photolysis of these compounds is referred to as ‘uncaging’. Uncaging is often employed in neurobiology research to release active neurotransmitters in synapses. Other commercially available caged compounds include nucleotides such as ATP and cAMP, ions such as calcium, and some macromolecules. In addition, proteins, peptides, DNA and RNA can also be caged.

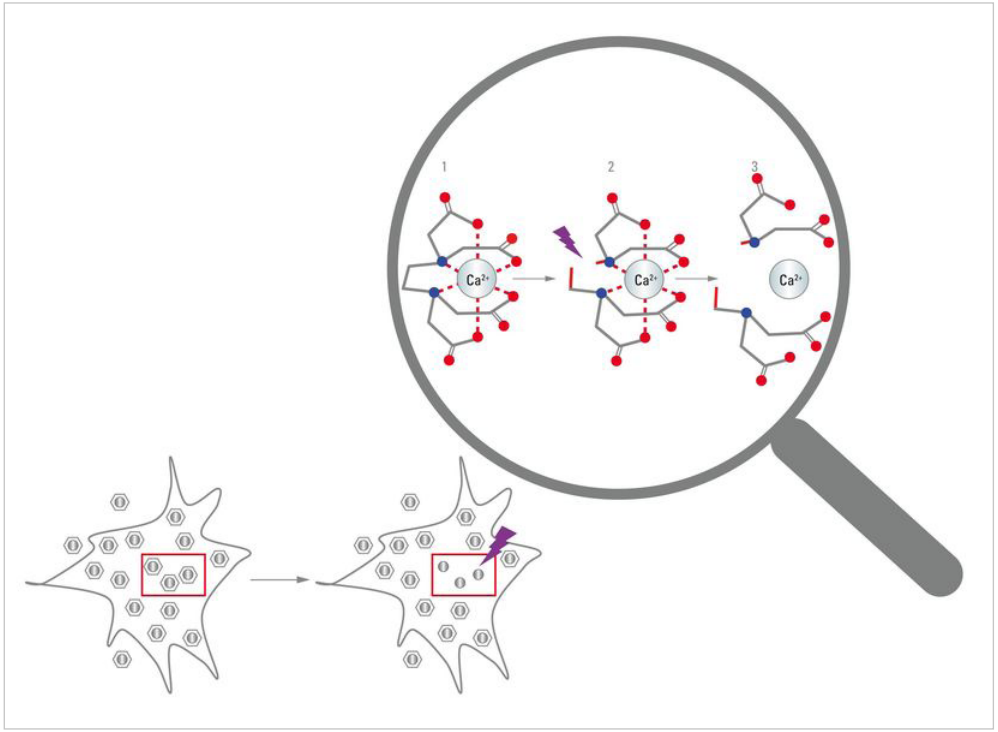


Figure 15: Uncaging: Chemically encapsulated bioactive substances such as Calcium ions are called “caged compounds”. With the help of UV light, the chelator can be damaged leading to the release of the caged compound.

Widefield Imaging and the Problem of Out-of-Focus Light

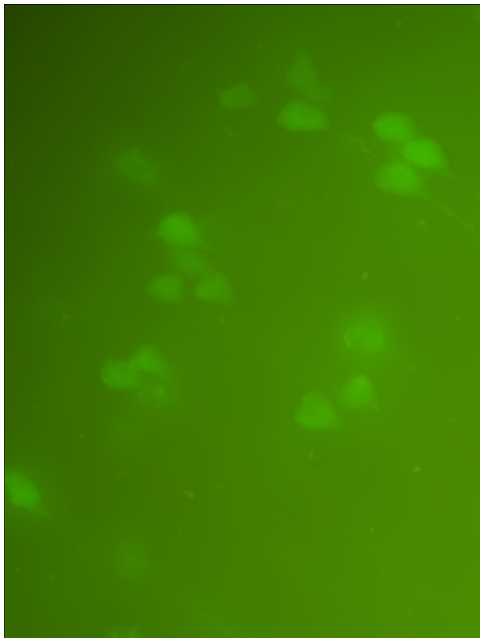
Widefield imaging is the technique whereby the entire specimen is exposed to a light source. Although widefield imaging requires less complex microscope systems, there are a number of inherent issues that can be problematic when using this technique for live cell imaging.

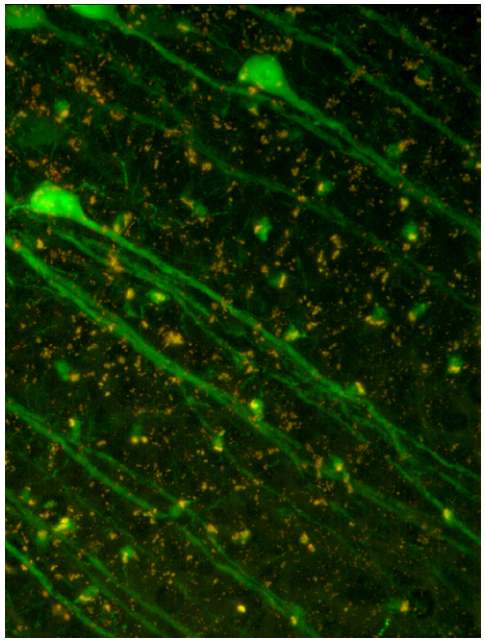
As the entire specimen volume is illuminated, image capture includes out-of-focus light above and below the focal plane that can result in blurring and a high background signal in fluorescence experiments, as well as decreased resolution and contrast.

There are a number of microscopy techniques and methods to overcome the problem of out-of-focus light, including;

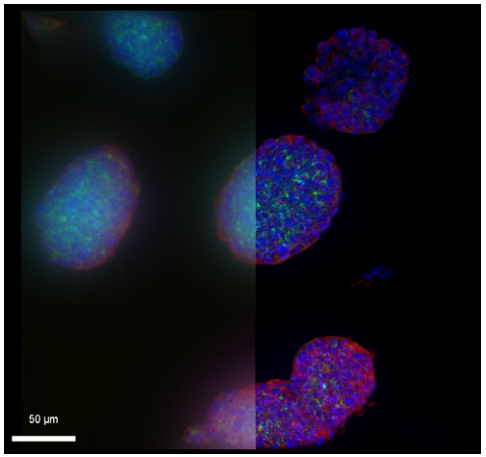
> **Deconvolution.** This is a post image acquisition technique that reassigns photons to their point of origin or removes out-of-focus light ("deblurring") from the final image. However, this method of image restoration relies on high-performance hardware and software, and deblurring can result in a loss of signal intensity and information.

> **Structured Illumination Microscopy (SIM).** This widefield technique uses a grid to produce patterned excitation of a sample to increase image contrast and produce optical sections. As the grid is moved to different positions, an interference pattern is produced between the excitation and emission signal. Algorithm processing produces a final image from the raw data. Although this technique can be used for live cell imaging, image processing can be time consuming and SIM can result in phototoxicity.





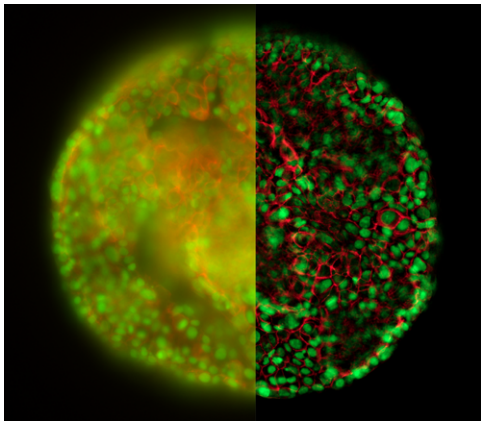
YFP mouse brain slices stained with GFAP-A647. Imaged with a THUNDER Imager Tissue shown on the right as opposed to standard fluorescence in the left image. Courtesy Dr. Hong Xu, University of Pennsylvania, Philadelphia (USA).



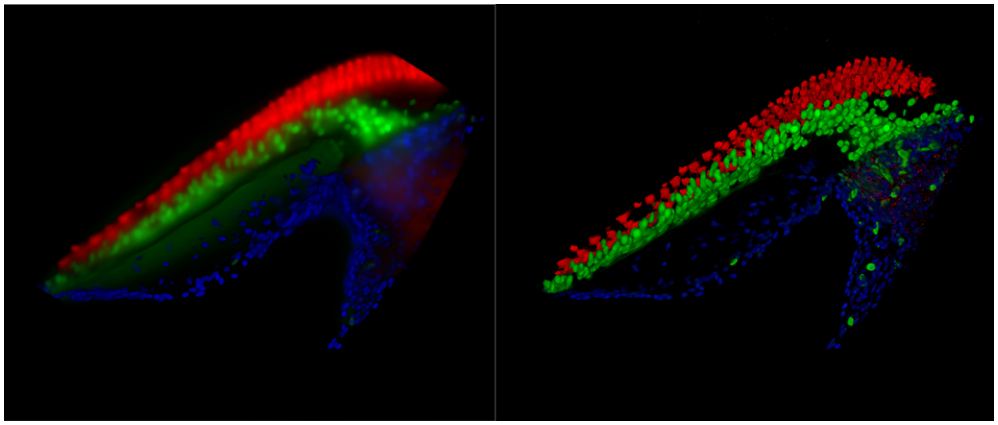
MIN6 cells grown as pseudoislets (pancreatic beta cells). DAPI (blue), Insulin (Alexa488, green), membrane receptor (Alexa594, red), phalloidin (Alexa647, white). Sample courtesy Dr. Rémy Bonnavion, MPI for Heart and Lung Research, Bad Nauheim (Germany).

Leica Microsystems THUNDER Imaging Systems

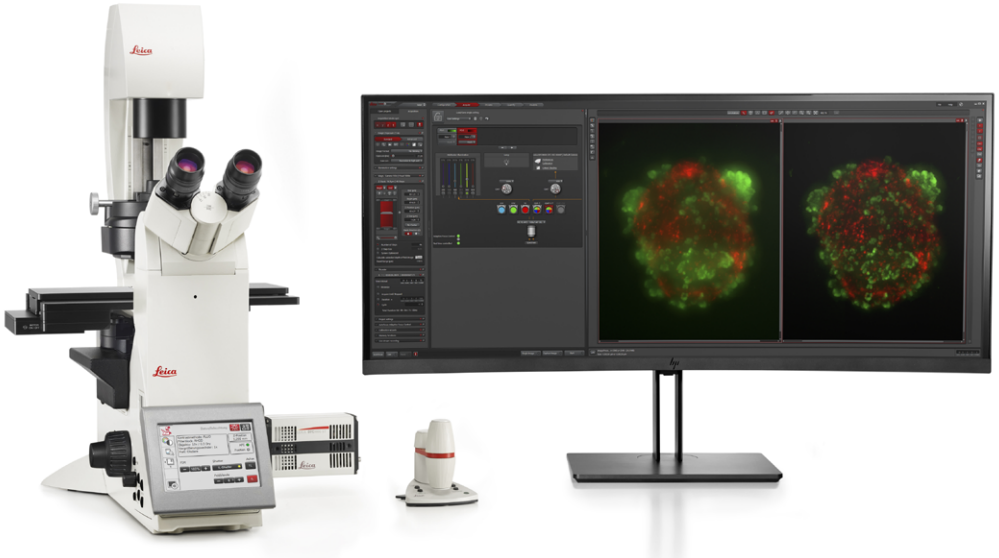
Leica Microsystems THUNDER Imaging Systems define a new class of instruments for high-speed, high-quality imaging of thick, 3D specimens. THUNDER is an opto-digital technology that uses the new Computational Clearing method to generate high resolution, high contrast images by removing the blur and out-of-focus light inherent to widefield imaging.



HeLa cell spheroid stained with Alexa Fluor 568 Phalloidin (Actin) and YOYO 1 iodide (Nucleus).



Cochlea cell – THUNDER Imager 3D Tissue Post-hatch day 7 chicken cochlea cells. 80 µm vibratome section, Myosin 7a – sensory hair cells, Sox2 – supporting cells Image courtesy Amanda Janesick, Stanford University, USA.

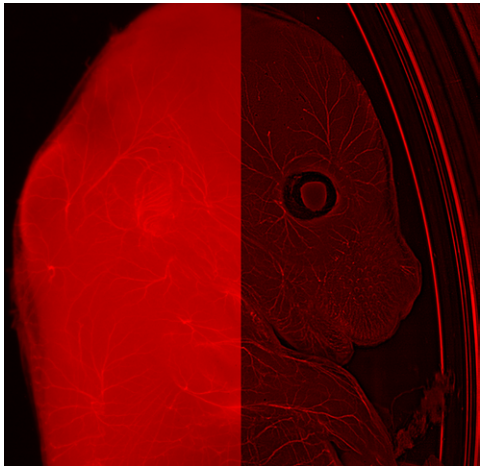


There are three variants of THUNDER imaging systems; THUNDER Imager Model Organism, THUNDER Imager Tissue and THUNDER Imager 3D Live Cell and 3D Cell Culture.

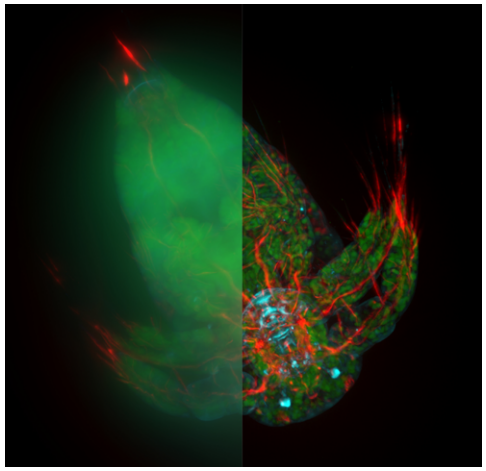
THUNDER Imagers enable the user to decode 3D biology in real time, even in thick samples such as whole organisms or tumor spheroids. The real time Computational Clearing method used by THUNDER is directly embedded in the image acquisition stream. By taking the size of the targeted specimen features into account, Computational Clearing efficiently differentiates between signal and background providing high quality images at very fast speed.

THUNDER Imager Model Organism

The heart of the THUNDER Imager Model Organism system is the Leica Microsystems M205 FCA fully motorized fluorescence stereo microscope. Combining a large field of view with high imaging quality, this system provides imaging solutions that were only previously possible with optical sectioning techniques. Whole model organisms can now be observed under physiological conditions with rapid acquisition of single blur-free images and complete Z-stacks. With three dedicated configurations, THUNDER Imager Model Organism can be optimized for large fields of view, sensitive imaging to capture faint signals, and investigation of rapid intracellular processes.



In this E12-14 mouse (wt sample), neurofilaments are stained in red to assess neuronal outgrowth. The mouse was cleared with the ScaleS reagent. Sample courtesy Yves Lutz, Centre d'imagerie, IGBMC (France).

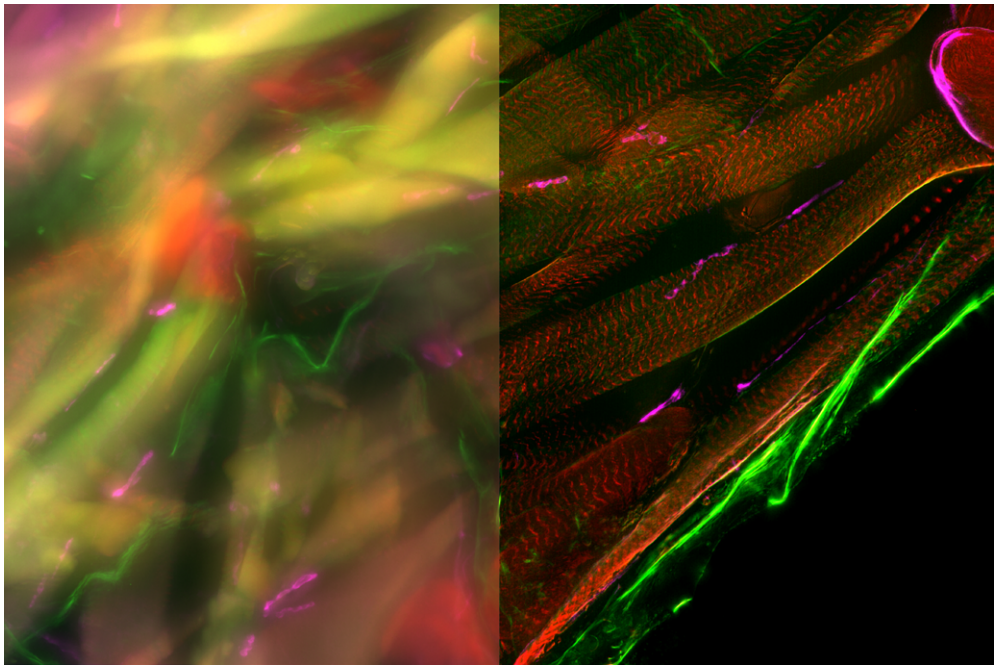


Cyclops sp. Nuclei (green), acetylated-tubulin (red), Serotonin (cyan). Image stack of a total volume of 332 x 332 x 84 µm³ with three colors and 305 z planes per color. Objective used was HC PL FLUOTAR 40x/1.30 OIL. Sample courtesy Dipl. Biol. Thomas Frase, University of Rostock, Allgemeine & Spezielle Zoologie, Institut for Biosciences, Rostock (Germany).

THUNDER Imager Tissue

Building on the advantages of widefield microscopy, THUNDER Imager Tissue with Computational Clearing captures real-time fluorescence images of thick tissue sections without the problems of out-of-focus light. Although widefield microscopes provide fluorescence efficiency, ease of use and fast imaging, blurring and background signal can obscure details – especially in thick sections. However, the THUNDER Imager Tissue with Computational Clearing removes out-of-focus blur in real time, enabling rapid acquisition of thick specimen images that show the finest cellular structures.

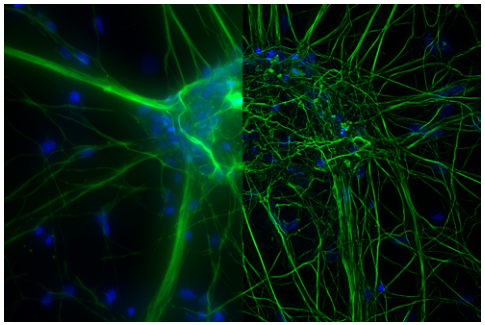
For brilliant imaging of single planes and fast overviews of tissue, the THUNDER Imager Tissue utilizes the benefits of the Leica Microsystems DM4 B upright digital research microscope. For fully automated recording of multi-color 3D images, the heart of the THUNDER Imager 3D Tissue is the Leica Microsystems DM6 B microscope – a fully automated system to acquire Z-stacks and decode 3D structures in real time.



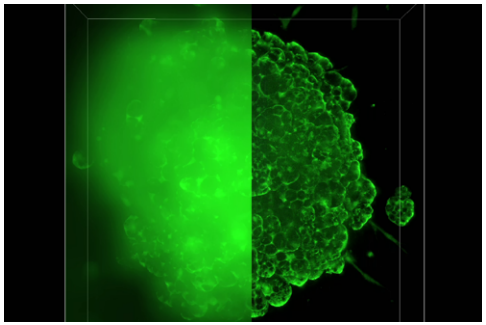
Drosophila third instar larval fillet labeled with AlexaFluor™647 for postsynaptic sites, AlexaFluor™555 conjugated to phalloidin, and AlexaFluor™488 labeling a subset of motor neurons.

THUNDER Imager 3D Live Cell and 3D Cell Culture

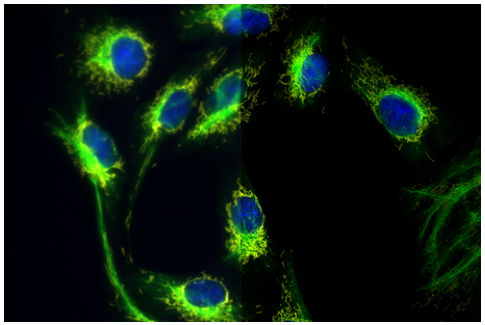
Live cell imaging experiments require optimal physiological conditions combined with low light intensity and the shortest exposure times to reduce phototoxicity. THUNDER Imager 3D Live Cell and 3D Cell Culture meet these demands with a high-end, multi-line LED source that has a small bandwidth optimized for excitation. The sensitive sCMOS camera captures meaningful image information, even with low illumination, and short exposure times due to a quantum efficiency of up to 82%. As the camera shutter is synchronized with the high-speed Lumencor LED, light exposure is reduced even further to minimize photobleaching of samples. With the incubator to ensure optimal physiological conditions for live cell culture, THUNDER Imager 3D Live Cell and 3D Cell Culture ensures that samples are observed under conditions at close to natural state.



Cultured Cortical Neurons. Green, beta-III-tubulin; blue, Nuclei. Image stack of 59 planes for a volume of 21 µm, was acquired using a THUNDER Imager 3D Cell Culture. Raw image compared to one image acquired using THUNDER Large Volume Computational Clearing. Sample Courtesy: FAN GmbH, Magdeburg (Germany).



Mouse lung organoids derived from alveola stem and progenitor cells. Sample courtesy Dr. Pumaree Kanrai, MPI for Heart and Lung Research, Bad Nauheim (Germany).



Cultured VERO cells stained with STAR488 Vimentin (green), STAR580 Tom20 (yellow), and DAPI (blue). Sample courtesy Abberior GmbH, Göttingen (Germany).

The Leica Microsystems DMi8 microscope platform of the THUNDER Imager 3D Live Cell and 3D Cell Culture enables automated multi-positioning and imaging across a number of sample carrier formats such as 8-chamber slides or 96-well plates. Accurately tracking the development of live cells can be problematic due to focus drift caused by temperature fluctuations and mechanical vibrations, as well as growth and morphological changes. However, THUNDER Imager 3D Live Cell and 3D Cell Culture overcome these problems due to:

- Reliable drift correction with the Adaptive Focus Control (AFC).
- Software autofocus that compensates for changes in specimen position.
- Reproducible Z-positioning with a precision of up to 20 nm (closed-loop focus).

With Computational Clearing embedded in the image acquisition stream, THUNDER Imager 3D Live Cell and 3D Cell Culture provide solutions for advanced 3D cell culture assays such as accurate and high-speed tracking of stem cells, or large sample volume imaging of spheroids and organoids.

To see more applications that benefit from the elimination of out-of-focus blur that clouds the view of thick samples when using camera-based fluorescence microscope, visit the THUNDER image gallery.

Leica Microsystems DMi8 S Inverted Microscope Platform

The DMi8 modular inverted microscope is the heart of Leica Microsystems DMi8 S platform and THUNDER Imager 3D Live Cell and 3D Cell Culture. It represents a complete solution for routine imaging and live cell research applications.

See More, with GPS for Your Cells: The LAS X Navigator Software Module

Leica Application Suite X (LAS X) is the one software platform for all Leica microscopes. It integrates confocal, widefield, stereo, super-resolution, and light-sheet instruments from Leica Microsystems. The Navigator is a module added to the LAS X software. It boasts a new, additional user interface, where the user gives carrier information (multiwell plate etc.), and the system will display the current field of view relative to the entire working area of the stage. Various standard carrier types are stored in the memory, and custom carrier profiles can also be created.

A mosaic of the entire working area (or whatever portion of it is required), or multiple mosaics (one per well, for multi-well plates) can be created and visualized as overview images, helping the user to find features of interest and designate areas for high resolution image acquisition.

Spiral scans can be used to search in the vicinity of the current location, and the user can rapidly zoom in and out or move to any stage location with the click of a mouse. Resulting mosaics can be assembled from any number of fields, the images of which can be stitched together at the end of the acquisition. Existing overview images of the same sample can also be used for pre-acquisition navigation, speeding up the process.

Drug screening assays are a good example where the LAS X Navigator can be of use, allowing the user to initially create a fast overview of an entire 96 well plate with a low magnification and, after identification of wells with cells of interest, proceed with high resolution image acquisition of the chosen candidates for a more detailed analysis. This approach not only saves time, but also protects cells from potential phototoxicity associated with slow manual searching.

Overview images are displayed in sample carrier templates for quick orientation and the viewing area can be increased up to 10,000x to quickly identify important details and set up high resolution image acquisition automatically using templates for slides, dishes and multiwell plates. Using the LAS X Navigator, any magnification, camera, detector, and contrasting method can be used in the same workspace. Results, as always, can be exported in a number of formats including TIFF or JPEG.

See Faster: Increase the Speed of Time Lapse Acquisition up to 5x with Intelligent Automation from the LAS XSynapse Advanced Sequencer

Simple live cell experiments, such as high frame rate time lapse recordings of a given channel, can be easily accomplished through the use of fast cameras or real-time controllers. However, more complex experiments that require control of components such as lens turrets, filter wheels, stage etc., are much slower in conventional systems. The time lag is due not only to the mechanical processes involved, but also because

the whole sequence is software-controlled: the software is the bottleneck of communication between all components.

This bottleneck is removed in the DMi8 S thanks to the LAS X Synapse advanced sequencer. In this configuration, the software only needs to give the start command for an experiment, and the LAS X Synapse real time sequencer takes over and directly communicates with all the components involved in the experiment, with no need for information exchange with the software in-between steps. The sequencer allows you to freely specify the behavior of connections, use both digital and analog signals, and set up the trigger signaling independently from the image acquisition with exact timings and full reproducibility.

This results in dramatically increased acquisition speed, up to 5x, with micro-second precision. This increase means that, depending on your needs, you can save time and/or achieve a higher temporal resolution for your time-lapse experiment, as the system runs at the highest possible speed for the given stage positions and for the light sources, cameras or other peripheral devices that are used.

External devices such as high speed filter wheels can be added for specialized applications and can be used in multi-position experiments with precise timing, without the bottleneck problems of conventional software control. Finally, if the user wishes to image a sample with a different contrast method, the microscope automatically sets the appropriate illumination settings, parfocality, brightness, and diaphragm position.

The LAS X Synapse advanced sequencer can make an essential difference to live cell imaging experiments that require a temporal resolution greater than the imaging possibilities achievable with conventional systems.

See the Hidden – Activate, Ablate, and Bleach in one Experiment

Adding the Infinity TIRF and Infinity Scanner to Leica Microsystems DMi8 S platform maximizes the versatility of the system. With this setup, the user can perform super-resolution microscopy, TIRF and several photomanipulation tasks, from optogenetics and photoswitching to FRAP and ablation, within one time-lapse experiment using up to five lasers.

Using the Infinity Port: The Advantage over the Conventional Infinity Optics Setup

In a compound microscope, the distance between the objective shoulder and the ocular shoulder is known as the “mechanical tube length” (Figure 16). This value was standardized to 160 mm by the Royal Microscopical Society in the 19th century. However, over the years the need to add more optical elements into the light path such as prisms and polarizers for DIC, or dichroics and filters for fluorescence microscopy meant a change in the effective tube length.

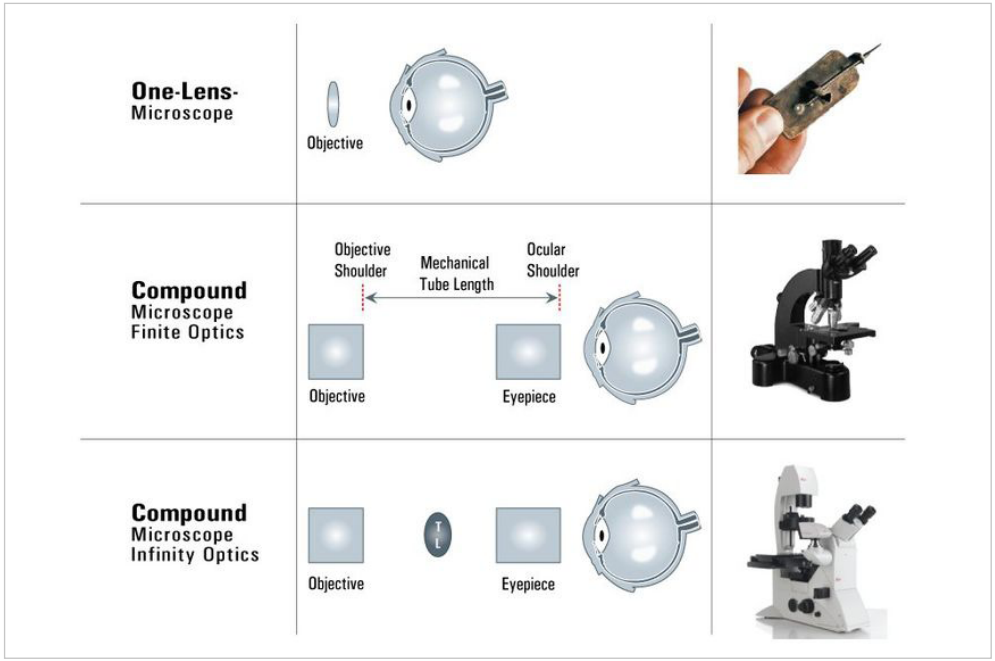


Figure 16: Top: Microscopy started with simple magnifying devices in the 16th and 17th centuries. The objective in this case is a single lens magnifying a specimen. Middle: A compound microscope with finite optics consists of a two-lens system. The objective magnifies the specimen, and the eyepiece magnifies the image produced by the objective. The distance between the objective shoulder and the ocular shoulder is called mechanical tube length. Bottom: A compound microscope with Infinity Optics holds an additional tube lens (TL).

Consequently, this introduced aberrations which had to be corrected with the addition of other components, but with the expense of diminished light intensities or increased magnification.

In the 1930s, the microscope manufacturer Reichert started to experiment with a setup called "infinity optics", which later became standard. In an infinity-corrected system the specimen is placed in the focal point of the objective, which projects a specimen image to infinity. In other words, light rays originating from a single point of the specimen are emitted from the objective in a parallel manner. Those derived from the center of the specimen (and the objective) run parallel to the optical axis. Those outside the center of the specimen run parallel to each other, at an angle to the optical axis. The space between the objective and tube lens is called the "infinity space" and the term 'infinity optics' refers to the production of parallel light rays after passing through the objective.

The virtual image produced by an infinity-corrected objective is captured by an additional lens to bring it to the front focal point of the eyepiece lens (Figure 17).

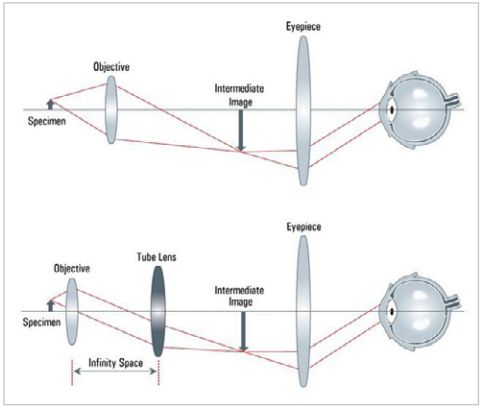


Figure 17: Top: A finite optical system consists of an objective and an eyepiece. The object is placed between the single and double focal point of the objective. The intermediate image produced by the objective is focused between the front focal point of the eyepiece and the eye piece. Users can view the image through the eyepiece. Bottom: In an infinity corrected system the specimen is placed in the focal point of the objective. In this case all rays coming from one point of the specimen are parallel after the objective, like an object placed in infinity. Rays coming from the specimen's center will leave the objective parallel to the optical axis (not shown). Rays coming from a single point of the specimen's periphery will leave the objective parallel to each other but not to the optical axis. The space between the objective and tube lens is called Infinity Space. Flat optical devices brought into this space will hardly affect the image since all rays coming from a single point of the specimen will experience the same optical influence. The tube lens forms a virtual image, which can be viewed through the eyepiece.

However, there is a limit to how much the infinity space can be extended by stacking modules into the microscope. Enlarging the imaging path between the objective and tube lenses will result in vignetting (a reduction in homogenous image brightness) and a reduction of the field of view, as off-center light rays will strike the tube lens at an angle.

There is an alternative way to enter the infinity space: instead of entering it through the imaging path between the objective and tube lenses, it can be accessed through the illumination path between the light source and the objective. Unlike the first approach, this second approach does not require stacking and eventual imaging path elongation, but instead allows access to multiple applications on the microscope by use of mirrors and beam splitters in the illumination path (Figure 18).

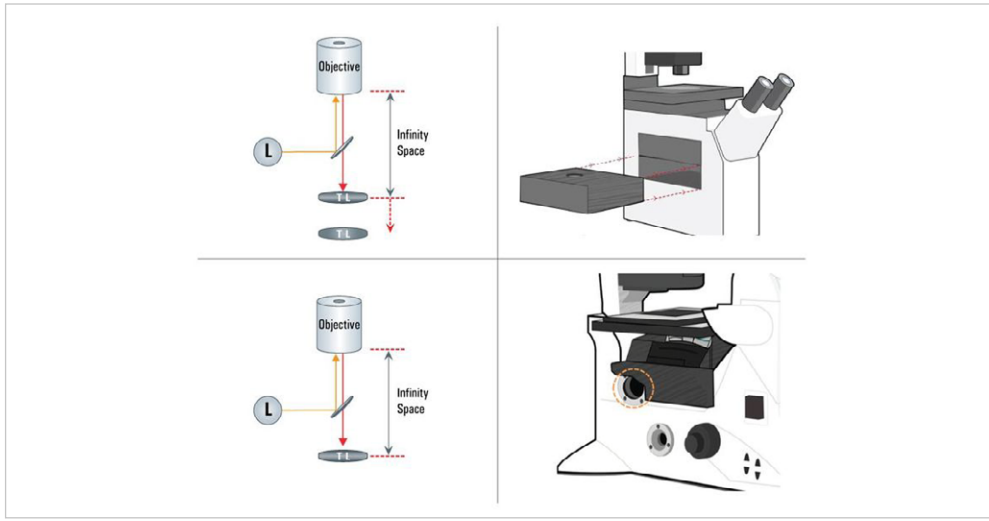


Figure 18: Access to the infinite light path of the microscope can be achieved either in the imaging path (top) between the objective and the tube lens (TL), or in the illumination path (bottom) between the light source (L) and the objective. The former approach requires stacking of the relevant module into the microscope and thereby elongates the imaging path. The second approach allows users access to multiple applications on the microscope by utilizing mirrors and beam splitters in the illumination path.

This solution, as implemented with the Leica Microsystems Infinity Port, not only preserves image quality by avoiding the insertion of extra elements in the light path, but also allows the connection of any kind of optical device required, by use of a suitable adaptor. The Leica Microsystems DMi8 S can be equipped with up to two Infinity Ports, allowing direct access into the fluorescent light path for addition of the latest in fluorescence technology, such as Infinity TIRF or the Infinity Scanner. In summary, the Infinity Port offers:

- Endless connection options through external couplings enabling the use of one or more of the special techniques described above.
- No filters and no photon loss.
- No extra elements or glass surfaces in the light path and no distortions.
- Full 19 mm field of view (FOV) maintained.
- No vignetting.

Infinity TIRF

The Leica Microsystems Infinity TIRF module offers versatile application possibilities with simultaneous multi-color EPI, Hi-Lo and TIRF illumination capability, as well as a high power illumination option for super-resolution applications (Figure 19).

The Infinity TIRF can be used with a large range of available widefield lasers for simultaneous multiple-color imaging. Plan APO corrected TIRF objectives offer the largest field of view on the market. In addition, the Infinity TIRF module offers azimuth-shifting to optimize the imaging conditions for each sample. Finally, the Infinity TIRF High Power (HP) system opens the door to multi-color single molecule resolution (down to 20 nm) with single molecule particle tracking, GSD, dSTORM and uPaint.

The Infinity TIRF module can also be added to the SP8 confocal system, further expanding its applications.

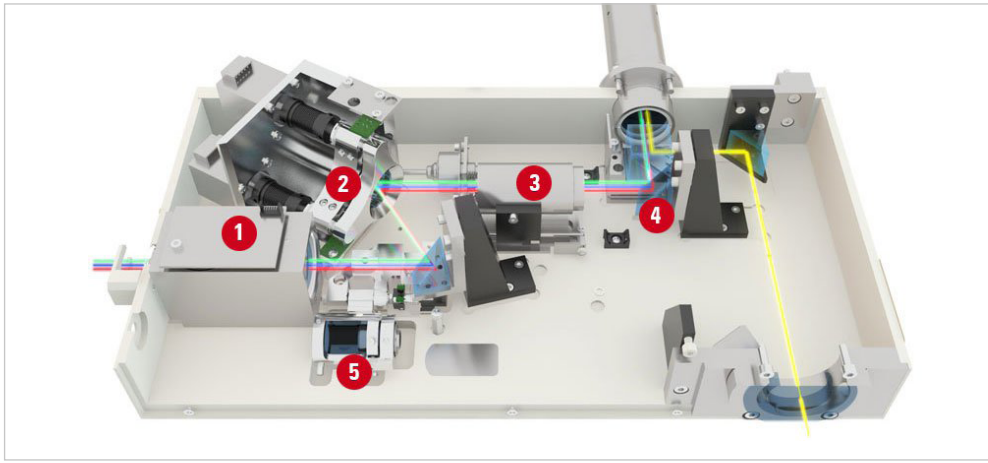


Figure 19: Leica Microsystems Infinity TIRF module. (1) TIRF Sensor: Detects the back reflected beam and enabling reproducible automated adjustment of TIRF penetration depth in every sample. (2) TIRF Scanner: Fine-tuning of TIRF penetration depth and adjustment of illumination direction (azimuth). (3) Movable collimator: Allows TIRF over full z-travel range with all Leica TIRF objectives. (4) Merge Optics: Combines a second illumination light path through Infinity TIRF module. (5) Beam Expander: Infinity TIRF HP module increases the power density on the sample for super-resolution imaging.

Infinity Scanner: the Ideal Multi-Spectral Photomanipulation Tool

The Infinity Scanner module of Leica Microsystems DMi8 S offers the ultimate multi-spectral photomanipulation tool for unparalleled application flexibility. Dual optical pathways allow the module to chromatically correct a wide range of lasers for multi-spectral photomanipulation applications, and the adjustable beam profile facilitates the flexible execution of experiments (Figure 20).

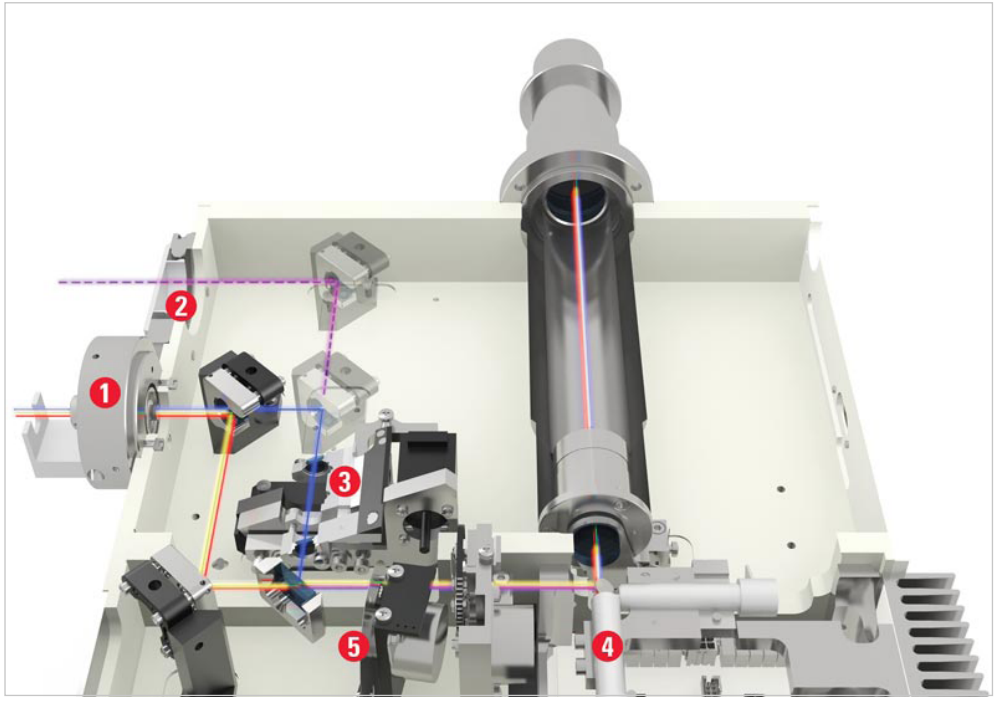


Figure 20: Leica Microsystems Infinity Scanner module. (1) Fiber Port: Coupling of laser fiber from Widefield Supply Unit, stand-alone lasers or 3rd party lasers. (2) Free Space Port: Fiber free direct laser coupling. (3) Vario-Optic: Corrects 350 to 800 nm wavelength lasers for parfocal scanning. (4) Galvo scanners: High speed X-Y scanning of laser beam. (5) Aperture: Adjustment of the scanner laser beam profile.

High speed vector scanning capabilities provide the user with precise control and to take advantage of the camera-based widefield system of the DMi8 S to capture the fastest cellular processes. Using the LAS X interface, multiple laser channels and techniques can be utilized within the same experiment, such as:

- FRAP, FLIP
- Acceptor bleaching
- Activation
- Switching
- Optogenetics
- Cutting
- Ablation
- Uncaging

Leica Adaptive Focus Control

Focus drift can be serious issue in live imaging, especially in long duration experiments. However, Leica Microsystems DMi8S is equipped with an LED light beam assisted Leica Adaptive Focus Control (AFC) that automatically maintains sample focus in real time, even when performing long-term time lapse experiments. The AFC is fully integrated into the LAS software. It can be used with over 85 objectives (dry, oil, water, glycerol) with magnifications ranging from 10x to 100x. The AFC can be used for imaging cells in plastic culture dishes and can be applied either in continuous mode, maintaining focus during time-lapse experiments and during XY movements of the stage, or on-demand, in Z-stacks, multi-position experiments, and in combination with image-based. The AFC actively refocuses with high speed and minimum phototoxicity.

Summary

In life sciences research, live cell imaging is an indispensable tool that allows the visualisation of cells in a state that is as close to in vivo as possible. Image capture of living cells enables you to fully understand and address your research questions regarding cell movement, growth, and dynamic processes.

Maintaining cellular viability and homeostasis is crucial to obtain meaningful data from long-term imaging experiments. Successful live cell imaging requires precise control of environmental factors such as temperature and pH.

Focus drift, out-of-focus light and phototoxicity can have detrimental effects on your imaging. It is therefore essential that your choice of imaging system negates these effects by removing out-of-focus light and correcting for drift, in combination with low light intensity and the shortest exposure times.

With the DMi8S and THUNDER Imagers, Leica Microsystems offer complete imaging solutions that are ideally suited to all of your live cell imaging applications.

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