Calcium imaging and imaging of other ions

In this Application Note we will provide an overview on different applications in ion fluorescence imaging.

There are a number of advantages of using fluorescence probes over other techniques. Depending on the experimental requirements different detector systems can be used to obtain high resolution information on the time course of events and/or on the spatial localisation of events.

High temporal resolution can be achieved using Photodiode based system. Changes in Ca2+ concentration can be measured in the order of milliseconds using a photodiode-based TILL Photometry setup.

High spatial resolution may be obtained using fast integrating CCD cameras which enable measurements over tens of milliseconds up to 200 Hz. This way additional spatial information of the intracellular environment can be obtained. TILL Photonics can provide high quality hardware and software packages in either of these fields.

Ion Imaging is a technique which typically combines microscopy with the use of ion sensitive fluorescent dyes in order to measure and visualize intracellular ion concentrations.

Ion sensitive dyes are fluorescent molecules which reversibly bind to specific ions. These dyes are very sensitive to any change in ion concentration.

A measure for the affinity of ion binding to the dye the dissociation constant (Kd) for that ion dye interaction. Different side groups at the molecule may in fluence the affinity of the dye to the specific ion (see figure 1).

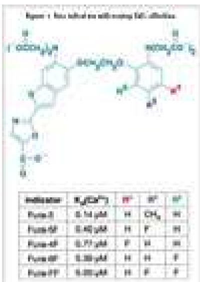

Figure 1

Binding of the ion causes conformational changes in the dye altering its fluorescence excitation and emission properties which can be used to report ion concentration.

Many different dyes are commercially available for measuring Ca2+ (or other ion) concentration and pH and it is important to select the most appropriate dye for your needs (www.probes.com).

Equipment for highest speed Imaging:

TILLvisION highest speed Imaging System consisting of:

- Polychrome V monochromator
- CCD Imago camera
- External control board for exact synchronization
- TILLvisION Imaging software

The TILLvisION highest speed Imaging System offers a perfect solution for:

- Highest speed imaging (up to 200 frames per sec)
- Ratiometric measurements
- Exact real time control of external devices using TTL or RS232 commands
- Multi-colour imaging with overlay function
- Post acquisition image analysis

Modular upgrade options are available for:

- Acqusistion of z-stacks
- 3 D and 4D rendering
- Deconvolution
- Dual Emission / FRET
- Particle Tracking

Photometry

- Polychrome V monochromator
- TILL photometry setup

Uncaging experiments combined with ion imaging:

- UV-Flash with specific 2 port epifluorescence condenser
- Polychrome V monochromator
- TILLvisION Imaging system or
- Photometry system

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Techniques and approaches in high speed calcium imaging

Introduction of ion sensitive dye to living cells

There are a number of possibilities to introduce a fluorescent dye into cells:

- ester loading
- low pH loading
- Electroporation
- Ionophoretic microinjection
- Pressure microinjection

For further reading please refer to:

A.J. Lacey (ed.): "Light Microscopy in Biology". A Practical Approach. 2 nd ed. Oxford University Press, 1989.
And to Molecular probes (www.molecularprobes.com)

Important considerations for live cell imaging:

Fluorescence intensity

The observed fluorescence emission is a relatively weak (factor 10^{-4} to 10^{-9}) compared to the power of the excitation light. Therefore it is very important to achieve efficient labelling of your cells.
The fluorescence intensity is directly correlated to the amount of fluorescent dye molecules in a certain volume.
In addition it is important to use a powerful and stable light source and to introduce the light into the microscope with high efficiency. In the TILL system, epifluorescence condensers are specifically designed for different microscope models to ensure efficient and homogeneous illumination of your sample.

Selection of the most suitable dye

Furthermore it is important to choose the correct fluorescent dye in terms of a good quantum yield, photobleaching and quenching. Please also refer to Fluorescent dye parameters.
Other factors such as cell permeability and life time in a living cell or toxicity are also important to consider as well as the most suitable dye loading method.

Bleaching

Too much excitation light for fluorescence imaging may cause cell damage and photobleaching of the dye. Photobleaching is a non-linear process and is in addition dependent of the dye used. In order to reduce photobleaching to a minimum you should illuminate your sample only when images are really acquired. This requires a system being able to accurately synchronize the illumination of your sample and the image acquisition with the camera.
If this synchronization is inaccurate, you will get artefacts due to a drift between the illumination and image acquisition timing. Most imaging system use the PC for synchronization. However the operating system of a PC will introduce delays of a few milliseconds, which, especially for short integration times, may lead to a drift in timing and cause artefacts.

Use optimal optics

If you use dyes excited in the UV you need to use specific objectives with a high UV transmission. In addition the NA (numerical aperture) of an objective determines the total amount of light passing through the objective. The higher the NA, the larger the cone of light focused. Since the light passes the objective twice, the NA influences the total light output by the factor of NA^2. Therefore High NA objectives are always preferable for fluorescence measurements.
DIC optics should be removed when measuring fluorescence in order to capture as much emitted light as possible and to keep exposure times low.

Suitable filter set

For each fluorescent dye a suitable filter set should be used. For new dyes it may be useful to run an excitation spectrum to find the best signal to background ratio for a given dichroic mirror. If you use dyes with different excitation wavelengths (e.g. FURA and GFP), you can also use one single filter set with a specific dichroic reflecting two or more wavelengths efficiently. Using the Polychrome V you can quickly switch between different wavelengths and also run an excitation spectrum.
If you have a specific configuration and you would like to know which filter set to use, please do not hesitate to contact us (info@till-photonics.com) to discuss the options you have.

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Light Sources

For fluorescence imaging usually Hg and Xe light sources are used.
Hg is usually used in standard epifluorescence illumination in most microscopes. It has a high intensity at certain wavelengths. The traditional fluorescent dyes have been designed to match these Hg lines. However with more complex applications new dyes are used which are excited at wavelengths where the Hg lamp does not have any peaks. Compared to the Hg lamp the spectrum of the Xe lamp shows a very even distribution of intensity over the wavelengths. This makes it more suitable for dyes excited at wavelengths between the Hg peaks.
With a Xenon lamp in a monochromator any wavelength in the visible spectrum can be chosen and the light output will have a comparable intensity over all wavelengths.
The Polychrome V is a monochromator combined with a Xenon light source.

High Speed Imaging

In order to speed up image acquisition to highest speed, the following factors have to be taken into account:

- the time needed to select an excitation wavelength
- the exposure time
- the read out time of the camera system
- With the Polychrome V, the wavelength can be changed within 2 ms.
- the time needed to select an excitation wavelength
- the exposure time (The exposure time can be minimized by on-chip binning by the factor of 4 and much more)
- the read out time of the camera system

The readout time of the camera system is dependent on:

- Resolution/binning
- Size of the area of interest
- The camera driver

In the selection of the excitation wavelength or the camera speed becomes the bottleneck in terms of speed.
After image acquisition, the image data has to be read out of the camera chip and saved before the next image can be taken.
In the TILLvisION high speed imaging system, one image can be acquired while the previous image is being processed and saved at the same time.
This enables a complete overlap of image acquisition and camera read out and makes use of the complete "dead time" which is otherwise lost during camera readout.
Therefore if you compare imaging cameras make sure you also compare the

exposure time at a given frame rate. For more details on the high speed modus in TILLvisION please contact us (info@till-photonics.com).

First measurements with living cells

If you are using new cells (or a new dye) and you don't know the expected response you can first test your cells and your dye with some simple experiments:

- Measure the intensity of your dye at different dye and ion concentrations.
- Test the response of your cells e.g. with ionophores to see the maximum response possible in your specific application.
- Usually it is useful to grow the cells (if possible) on cover slips in order to make them stick to a surface. This coverslip can be easily used for imaging later on.

Fluorescent dye parameters

Important dye parameters to consider are:

- specificity for the ion of interest
- dissociation constant Kd
- excitation and emission spectrum
- fluorescence intensity
- free Ca^{2+} range
- cell permeability (available as a AM ester)
- efficiency of Photon absorption
- quantum yield (ratio of absorbed and emitted photons)
- sensitivity to photobleaching (chemical destruction of the dye during illumination)
- quenching (deactivation of excited dye molecules without the emission of any radiation)

For more information on fluorescent ion sensitive dyes, please refer to the Molecular probes Handbook.

There are three major classes of ion-sensitive dyes:

1. single excitation dyes
2. dual excitation ratiometric dyes
3. dual emission ratiometric dyes

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1. Single excitation dyes

With single wavelength dyes the intensity of the fluorescence emission spectrum increases in proportion to free ion concentration.
The problem with using a single wavelength dye is that it is difficult to distinguish between differences in ion concentration and variations in the dye brightness caused by factors such as dye concentration, dye photobleaching and dye leakage from a cell.
This makes absolute measurement of ion concentration with single wavelength dyes difficult. Examples are dyes of the Fluo-family, such as Fluo 3.

2. Dual excitation ratiometric dyes

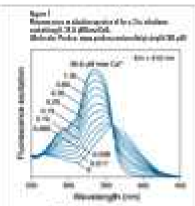

Figure 2

These dyes may be excited successively at two different wavelengths. The emitted light increases with increasing [Ca]I at one excitation wavelength, and decreases at a second wavelength (see figure 2). A prominent example is FURA 4.

The following table summarize some dual excitation ratiometric dyes:

Dual Excitation – Single Emission Dyes				
Ion	Probe	Ex 1 Wavelength (nm)	Ex 2 Wavelength (nm)	Em Wavelength (nm)
Calcium	Fura-2	340	380	510
Magnesium	Mag-Fura-2	340	380	510
Magnesium	Mag-Fura-5	340	380	500
pH	BCECF	440	490	530
pH	SNAFL-1	514	550	600
Sodium	SBFI	340	380	505
Potassium	SBFI	340	380	505

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3. Dual emission ratiometric dyes

These dyes are excited at one wavelength and fluorescence emission at two longer wavelengths. With indo-1 and cSNARF-1, the two emission wavelength correspond to the ion binding and free forms of each dye. As the concentration of free Ca^{2+} or protons increases, there is a shift in the emission spectra of Indo-1 and cSNARF-1 respectively, to shorter wavelengths of the ion binding forms of these dyes. Please refer to the Molecular Probes Homepage (www.molecularprobes.com) for more information on Indo-1 and cSNARF-1.

Application of the TILL Photonics Imaging System

- Multi colour ratio imaging
  Required components:
  - Polychrome V (as a monochromator it gives ultimate flexibility to choose excitation wavelength from 320 nm – 680 nm) TILLvisION
  - Camera (IMAGO VGA; QE or EM)
  - Control unit for precise timing and synchronisation for illumination and image acquisition

  E.g. for simultaneous measurement of calcium and pH

- Fast time lapse measurements
  Required components:
  - Polychrome V (as a monochromator it gives ultimate flexibility to choose excitation wavelength from 320 nm – 680 nm)
  - TILLvisION
  - Control unit for precise timing and synchronisation for illumination and image acquisition
  - IMAGO VGA Camera
    - IMAGO VGA Camera is used for fastest acquisition
      - Up to 30 full frames per second
      - Up to 200 sub frames per second

- Multi color GFP
  Required components:
  - Polychrome V (as a monochromator it gives ultimate flexibility to choose excitation wavelength from 320 nm – 680 nm)
  - TILLvisION / evtl. Basic Imaging Software

- IMAGO QE Camera (QE recommended for higher sensitivity)
- TILL GFP-Filtersets
    - Specially adapted to the monochromator
    - CFP/YFP double labelling
    - Other filter combinations on request

- Fluorescence overlay
  Required components:
    - Polychrome V (as a monochromator it gives ultimate flexibility to choose excitation wavelength from 320 nm – 680 nm)
    - TILLvisION / evtl. Basic Imaging Software

    - IMAGO VGA or QE Camera
    - optionally IR-Illumination
        - Transmission images can be overlayed with fluorescence images
        - For fast switching between transmission and fluorescence a high power IR-LED illumination is available
        - IR-Illumination is controlled by TILLvisION and can be used in combination with the microscopes halogen illumination

- Digital Video Imaging and photography
    - Live mode with the IMAGO VGA camera is fast enough to replace a video camera for application like e.g. observing the patch pipette during seal formation

- Automated microscopy
    - Polychrome V (as a monochromator it gives ultimate flexibility to choose excitation wavelength from 320 nm – 680 nm)
    - TILLvisION
    - IMAGO VGA, QE or EM
    - iMIC (or other motorized microscopes)
    - Motorized stages (not required with iMIC)
    - Piezo z-drive for fast and precise z-movement (not required with iMIC)

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- Multi emission measurements (e.g. FRET)
    - Polychrome V (as a monochromator it gives ultimate flexibility to choose excitation wavelength from 320 nm – 680 nm)
    - TILLvisION
    - IMAGO VGA, QE or EM camera
        - Filter wheel (Sutter lanmda 10-2)
            - Software or manually controlled filter wheel in the emission path
            - Fully integrated into the protocol editor
            - Up to 9 different emission filters can be used
            - Suitable also for FRET experiments
        - Fluorescence Resonance Energy Transfer (FRET)
            - Polychrome V (as a monochromator it gives ultimate flexibility to choose excitation wavelength from 320 nm – 680 nm)
            - TILLvisION
            - QE or EM camera
            - MicroImager for simultaneous dual emission image acquisition
                - Acquires 2 or 4 images simultaneously
                - Very light efficient
                - No moving parts
            - Alignment and analysis supported by special software module
                - Allows real simultaneous emission measurements by projecting images on different regions of the camera chip. Suitable e.g. for CFP/YFP FRET

- Particle tracking and motion analysis
    - Polychrome V (as a monochromator it gives ultimate flexibility to choose excitation wavelength from 320 nm – 680 nm)
    - TILLvisIOn
    - Control unit for precise timing and synchronisation for illumination and image acquisition
    - IMAGO VGA, QE or EM camera
    - Tracking Module (TILLvisTRACK)
        - Automated multi-particle detection
        - Automated dynamic object tracking
        - Quantitative evaluation and 3D visualization
        - Colocalization analysis

For advanced Live Cell imaging and quantitative analysis of dynamic cellular processes

- Deconvolution
  - Polychrome V (as a monochromator it gives ultimate flexibility to choose excitation wavelength from 320 nm – 680 nm)
  - TILLvisION
  - Control unit for precise timing and synchronisation for illumination and image acquisition
  - IMAGO VGA, QE or EM camera
  - Piezo z-drive for fast and precise z-movement
    - Piezo based objective positioning
    - To aquire z-stacks with 100 nm resolution
    - < 20 ms positiong time
  - Deconvolution module (TILLvisDECO)
    - Excellent resolution and speed
    - Acquisition of the point spread function
    - Calculation of the filter kernel
    - Fast deconvolution (within minutes)

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