



FLUORESCENCE FOUNDATION

TIRF microscopy
SPARTACO SANTI

Abstract

Total internal reflection fluorescence microscopy (TIRFM) exploits the unique properties of an induced field in a limited specimen region immediately adjacent to the interface between two media having different refractive indices.

This surface electromagnetic field, called the 'evanescent wave', can selectively excite fluorescent molecules in the liquid near the interface. In practice, the most commonly utilized interface in the application of TIRFM is the contact area between a specimen and a glass coverslip or tissue culture container.

TIRF examination of cell/surface contacts dramatically reduces background fluorescence from fluorophores either in the bulk solution or inside the cells (i.e. autofluorescence and debris). Moreover, because TIRF minimizes the exposure of the cell interior to light, the healthy survival of the culture during imaging procedures is much enhanced relative to standard epi- (or trans-) illumination.

In general, total internal reflection illumination has potential benefits in any application requiring imaging of minute structures or single molecules in specimens having large numbers of fluorophores located outside of the optical plane of interest, such as molecules in solution in Brownian motion, vesicles undergoing endocytosis or exocytosis, or single protein trafficking in cells.

An ideal candidate for application of the technique is the study of neurotransmitter release and uptake from single vesicles in primary culture of neurons and astrocytes.

Background of TIRF microscopy



the phenomenon



Refractive Index

of a material is the factor by which the phase velocity of electromagnetic radiation is slowed in that material, relative to its velocity in a vacuum.

Refraction

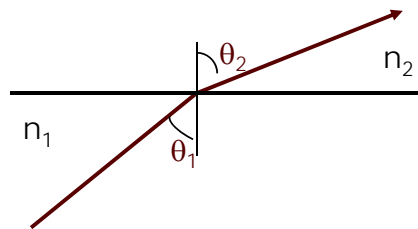
is the change in direction of a wave due to a change in the velocity of the wave

Snell's law

Is the formula used to calculate the refraction of light when travelling between two media of differing refractive index. When light hits a surface with angle θ_1 against the vertical axis, then a part will be reflected, the rest is getting refracted and enters the medium with the angle θ_2 against the vertical axis



Willebrord Snell
1580-1626

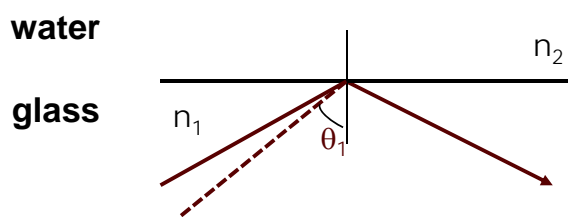


$$n_1 \sin(\theta_1) = n_2 \sin(\theta_2)$$

θ = angle of incidence, angle of refraction
 n = refractive indices

TIR

When $n_1 > n_2$ and the angle of incidence of light (θ) is wider than the critical angle (θ_c) the transition into the medium with lower density is not possible and **Total Internal Reflection** occurs



$$\theta_c = \sin^{-1} (n_2/n_1)$$

$$n_1 = 1,518 \text{ (glass)}$$

$$n_2 = 1,36 \text{ (water)}$$

$$\theta_c = \sin^{-1} (1,37/1,518)$$

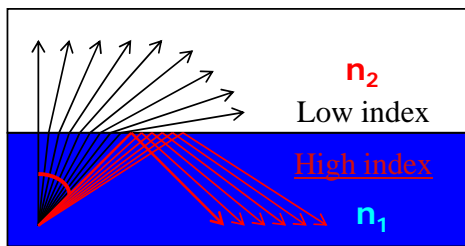
$$\theta_c = 63,95^\circ$$

n_1 = higher refractive index / higher optical density
 n_2 = lower refr. index / lower optical density

TIR conditions

interface of two media with different refraction indices

$$n_1 \sin(\theta_1) = n_2 \sin(\theta_2)$$



$$\theta_c = \sin^{-1}(n_2/n_1)$$

$$n_1 = 1,518 \text{ (glass)}$$

$$n_2 = 1,36 \text{ (water)}$$

$$\theta_c = \sin^{-1}(1,37/1,518)$$

$$\theta_c = 63,95^\circ$$

it is easily verified that: $\theta_1 = 0 \Rightarrow \theta_2 = 0$

when $\theta_1 > \theta_c$, no refracted ray appears, and the incident ray undergoes **total internal reflection** from the interface.

Evanescent wave



Once in Total Internal Reflection, an evanescent wave is generated: this evanescent field is identical in frequency to the incident light; it is an electromagnetic wave that **decays exponentially** with distance from the interface at which it is formed

Depth of penetration of the evanescent wave decreases exponentially with the distance to the interface and depends on:

- Light wavelength
- Angle of incidence
- Refractive indices
An evanescent wave (tunnel effect) travels into the medium with lower density (i.e. adherent cells)

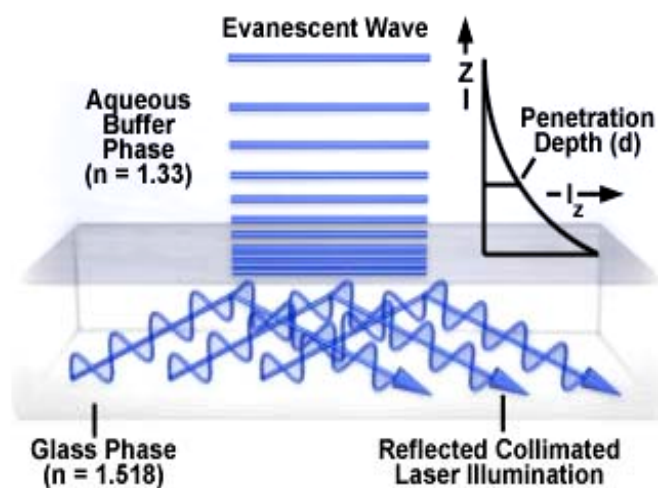
Evanescent wave

Once in Total Internal Reflection, an evanescent wave is generated: it is an electromagnetic wave that **decays exponentially** with distance from the interface at which it is formed

$$I(z) = I(0)e^{-\frac{z}{d}}$$

with

$$d = \frac{\lambda}{4\pi} \left(n_{\text{sample}}^2 \sin^2 \theta - n_{\text{oil}}^2 \right)^{-1/2}$$



Evanescent wave

Once in Total Internal Reflection, an evanescent wave is generated: it is an electromagnetic wave that **decays exponentially** with distance from the interface at which it is formed

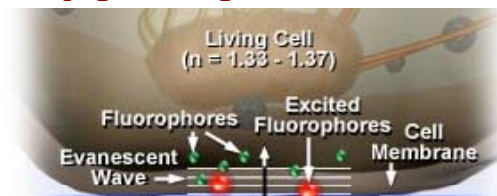
Distance (nm)	Relative intensity
1	0.99
10	0.92
100	0.43
1000	0.0002

Energy of evanescent field
 $E(z) = E(0)\exp(-z/d)$

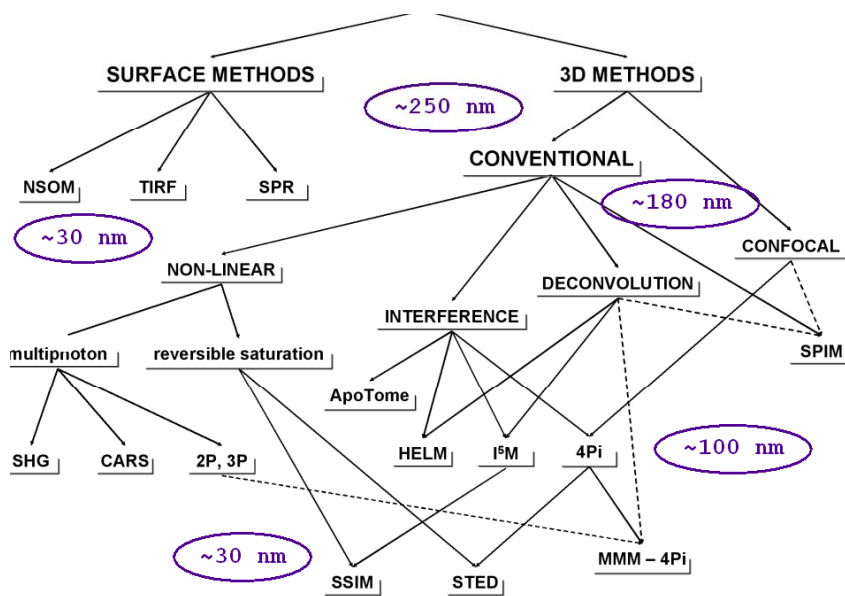
In microscopy

the induced evanescent wave selectively illuminate and excite fluorophores in a restricted specimen region immediately adjacent to a glass-water (or glass-buffer) interface.

minimal photodamage
very good signal-to-noise ratio

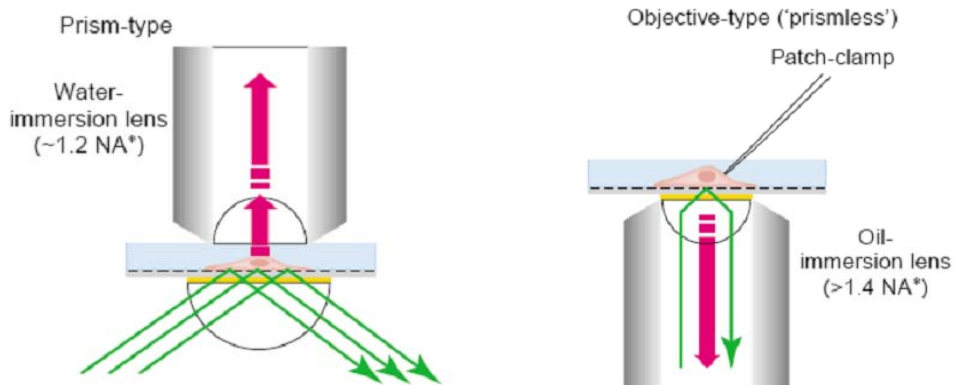


TIRF is an optical surface method



Garini et al, Curr Opin Biotech 2005. 16, 3-12

Prism/Objective-type TIRF



Pros and Cons

- | | | |
|---|--------------------|---|
| - | penetration deep | + |
| - | access to sample | + |
| - | system setup | + |
| - | laser safety | + |
| + | objective NA > 1.4 | - |

from technical point of view we need:

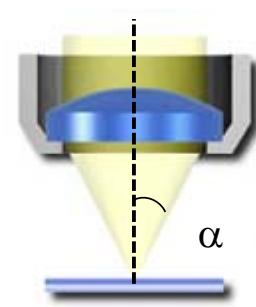
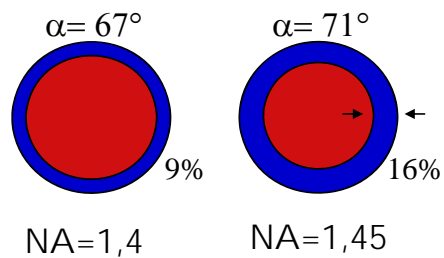
- high numerical aperture objective, $NA > 1.45$

$$\theta_c = \sin^{-1} (n_2/n_1)$$

$$n_1 = 1,518 \text{ (glass)}$$

$$n_2 = 1,33 \text{ (cells)}$$

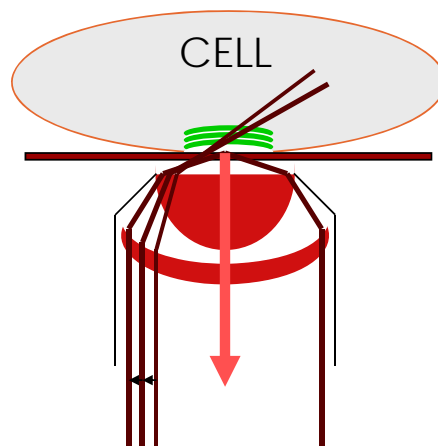
$$\theta_c = \sin^{-1} (1,37/1,518) = 61^\circ$$



$$NA = n \sin \alpha$$

from technical point of view we need:

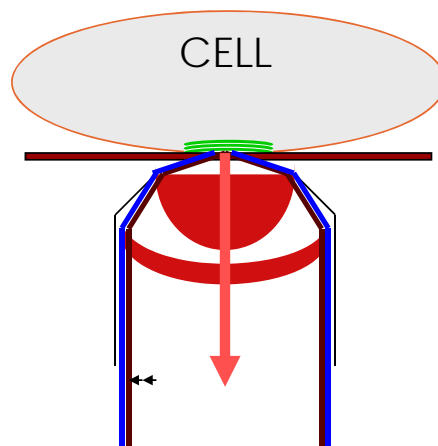
- high numerical aperture objective, $NA > 1.45$
- **specific condenser**



Critical angle
high penetration depth

from technical point of view we need:

- high numerical aperture objective, $NA > 1.45$
- **specific condenser**



Critical angle
lowest penetration depth

from technical point of view we need:

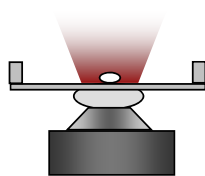
- high numerical aperture objective, $NA > 1.45$
- specific condenser
- **laser beam**

4 solid state diode laser 405 (25mW), 488 (10 mW), 561(20 mW), 635nm(18 mW)
AOTF control for switching wavelenghts (1 ms) and intensity
All lines through one multimode fibre

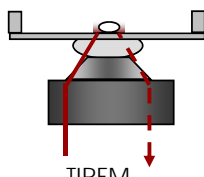


Illumination and Z resolution

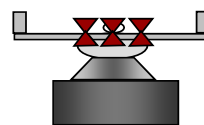
Petri Dish
Oil
Objective



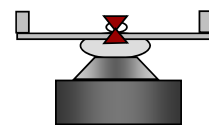
WFM
1,13 μ m



TIRFM
~ 0,1 μ m

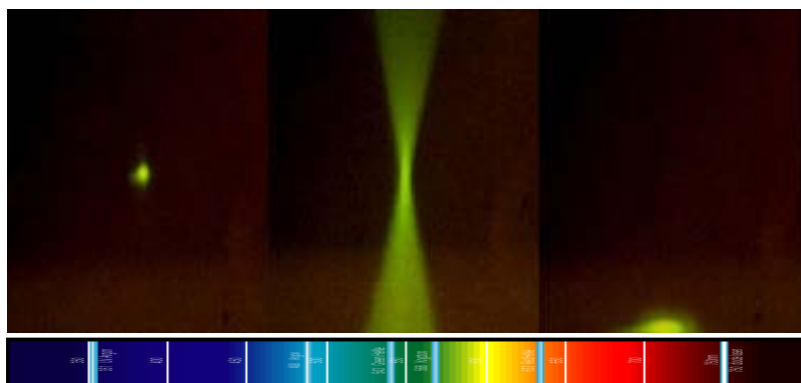


SDCM
0,84 μ m

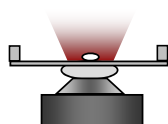


LSCM
0,56 μ m

Comparison 2PE-CLSM-TIRFM excitation



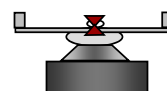
Speedofacquisition



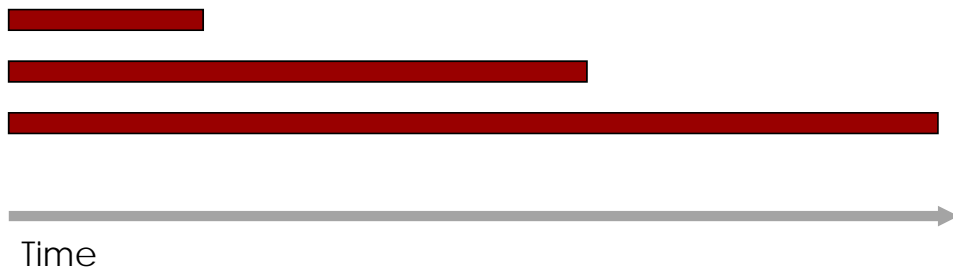
WFM/TIRF



SDCM



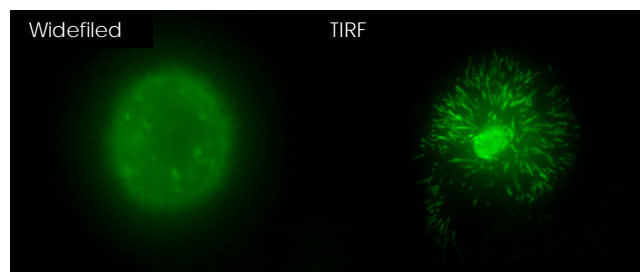
LSCM



Time

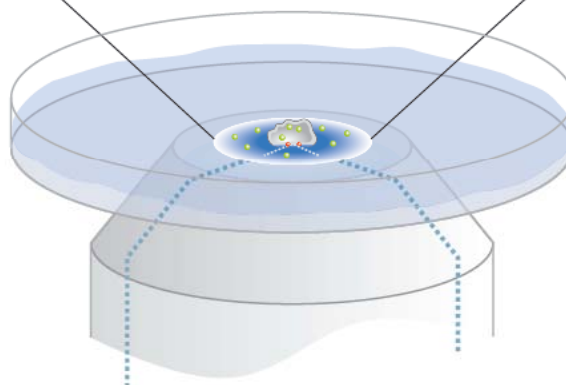
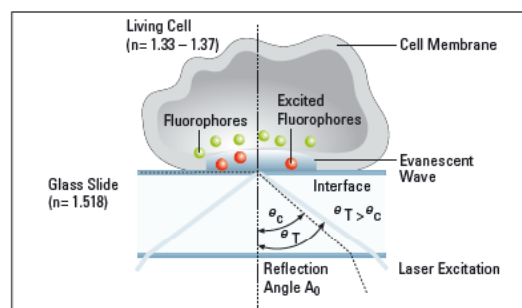
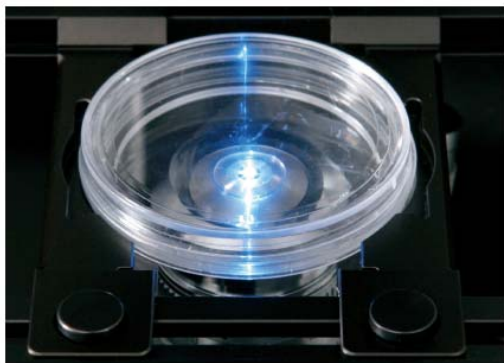
Advantages of TIRF microscopy

- Higher Z-resolution (80-300 nm)
- High signal to noise ratio
- High contrast in Fluorescence (no out of focus information)
- Less bleaching and light stress for living cells
- Good method to combine kinetic studies with local information in living samples

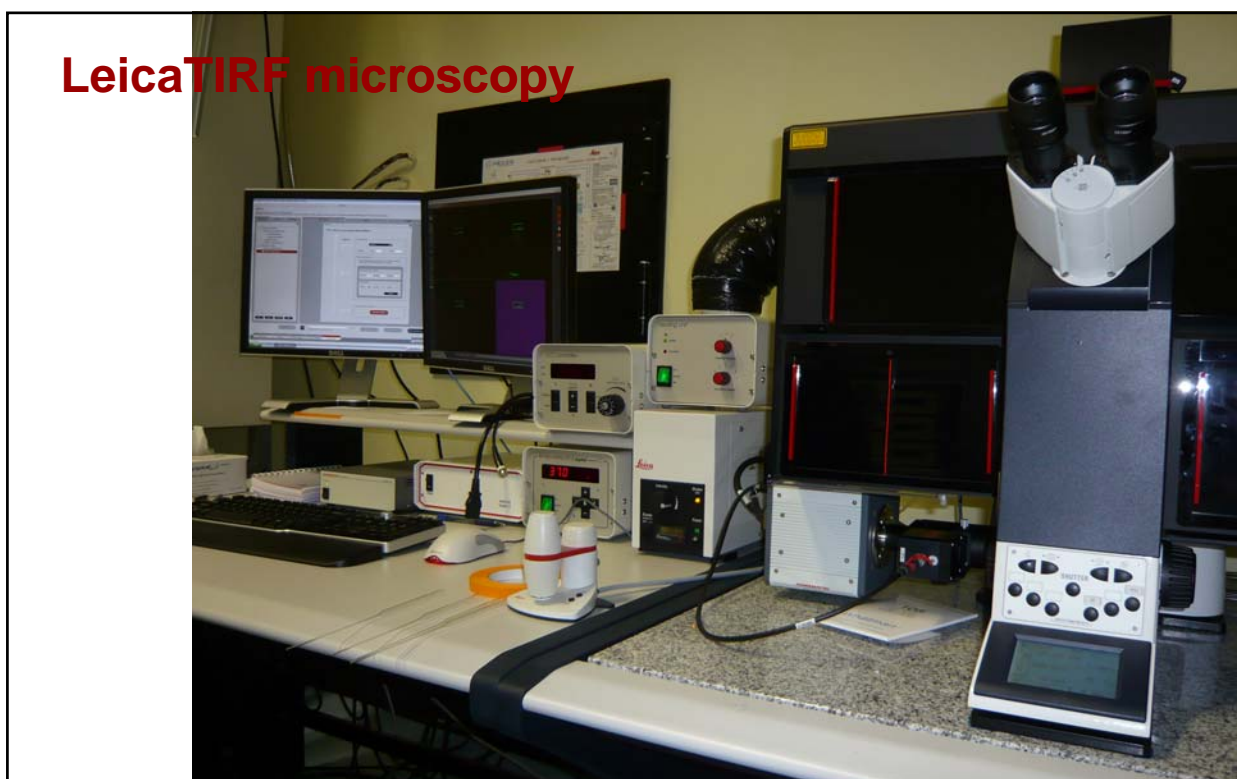


TIRF microscopy setting

- Sample in medium/water
- TIRF oil Immersion objective
- Glass interface

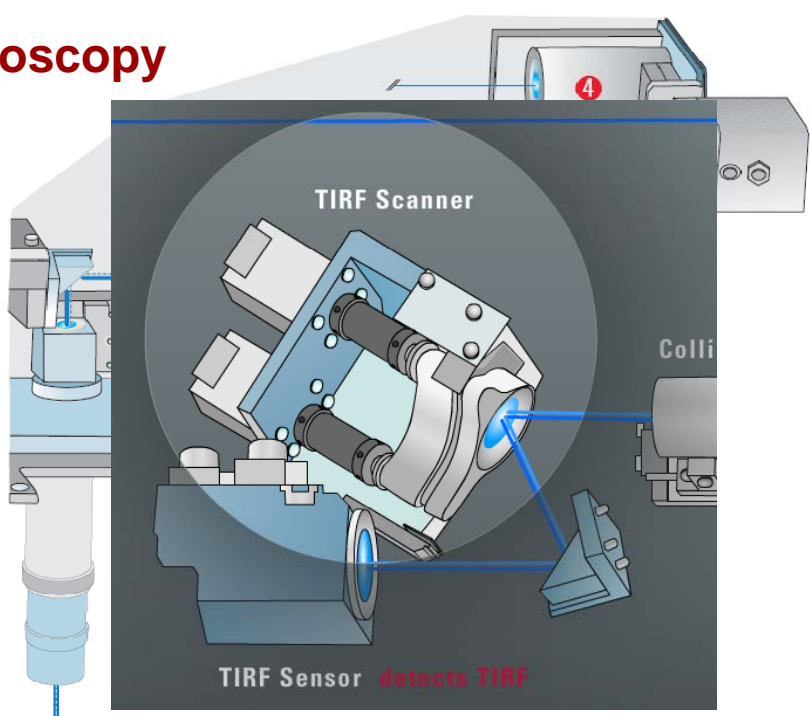


LeicaTIRF microscopy



Leica TIRF microscopy

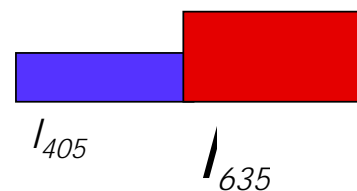
1. **TIRF sensor** (refractive index of sample, position of reflected laser beam)
2. **TIRF scanner** (definition of penetration depth, critical angle, automatic correlation of penetration depth according to wavelength, direction of evanescent field)
3. **Collimator** (Z focus control of laser beam)
4. **Widefield fluorescence excitation**



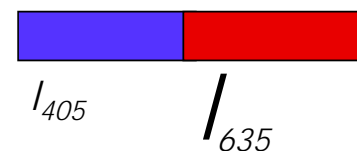
Leica TIRF microscopy

- Setting of penetration depth: 70-250 nm
- Automatic correlation of penetration depth of different laser beam wavelengths: *changing wavelength from lower to higher λ leads to a higher penetration depth and therefore to different signals in the experiment*

Different laser beam wavelengths produce different penetration depths and different volume detected



The TIRF module automatically corrects the differences in the penetration depths adapting the critical angle in relation with the laser beam wavelengths.



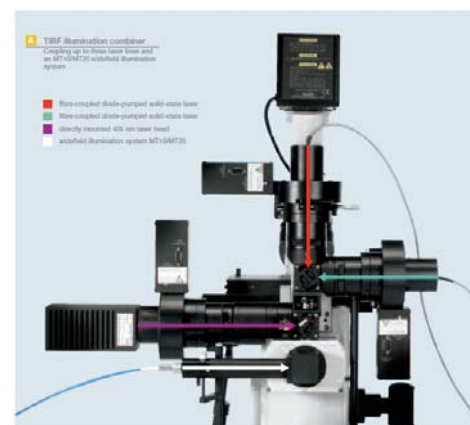
Zeiss TIRF microscopy

- The apochromatically corrected optical system allows the use of any laser wavelength between 405 and 650 nm with a common beam focus.
- The motorized TIRF slider for reproducible angle settings: a penetration depth resolution of 5 nm within the middle range of TIRF angles
- Simultaneous detection



Olympus TIRF microscopy

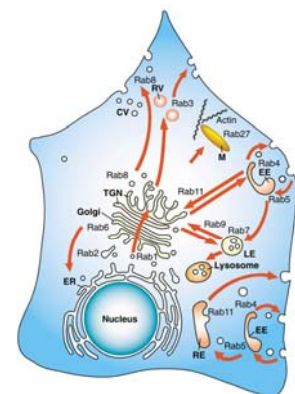
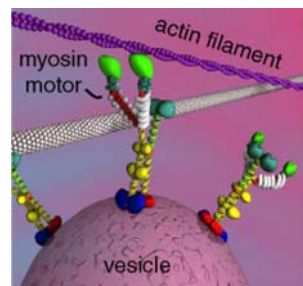
- TIRFM illumination combiners provide individual ports for each laser to be coupled via a corresponding single-line fibre. Each laser beam position can thus be adjusted to match the corresponding filter cube.
- Simultaneous detection



Applications

Membrane research

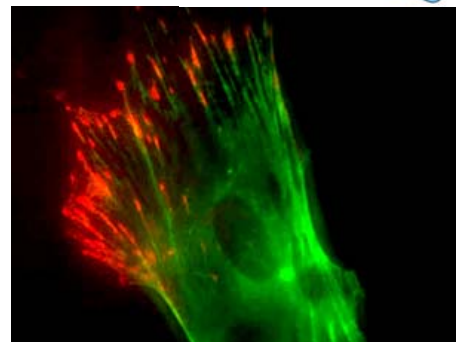
- Diffusion of molecules (e.g. Syntaxin)
- Kinetic of transporters
- Membrane fusion
- Cell/Cell interaction
- Cytoskeletaldynamics



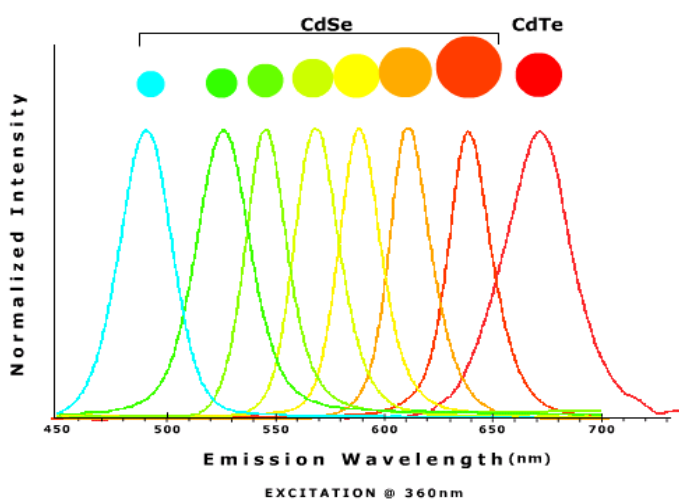
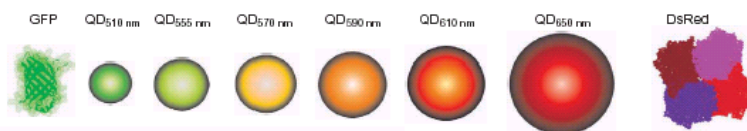
Vesicle transport

- Understanding of transport processes
- Localization of molecules
- Endocytosis and Exocytosis

Single molecule detection



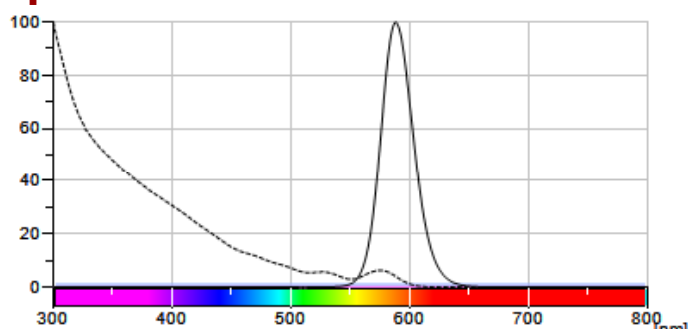
Quantum Dots



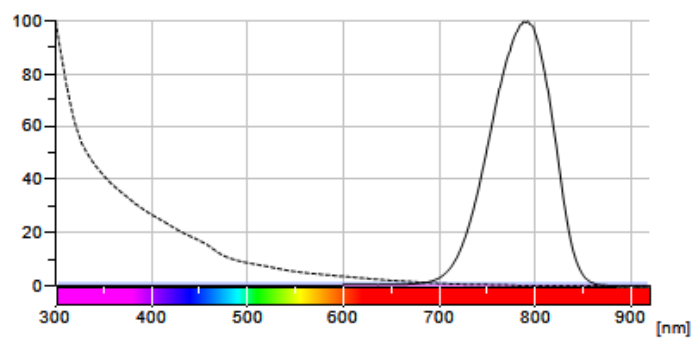
Excitation spectra



Qdots 585

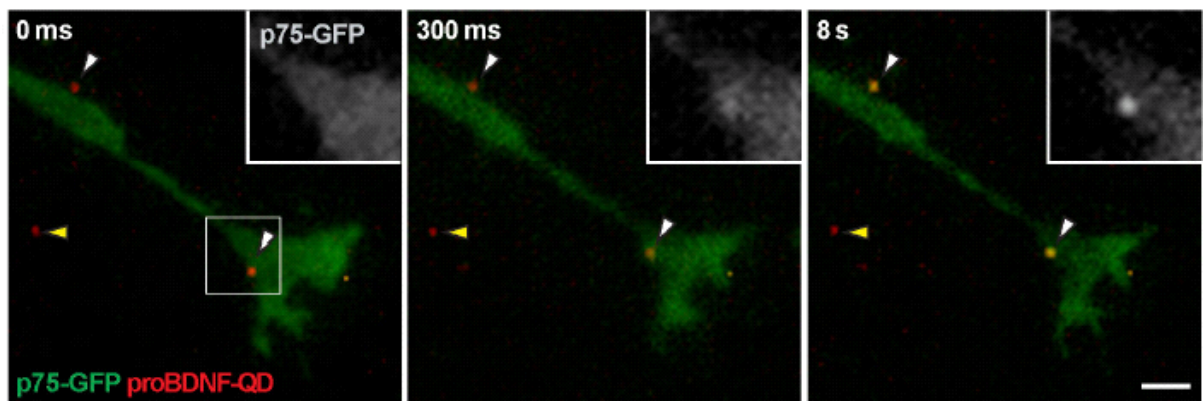
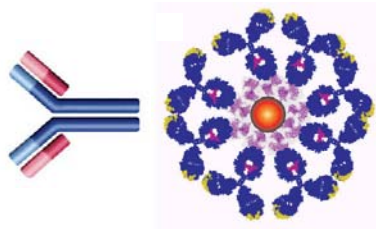


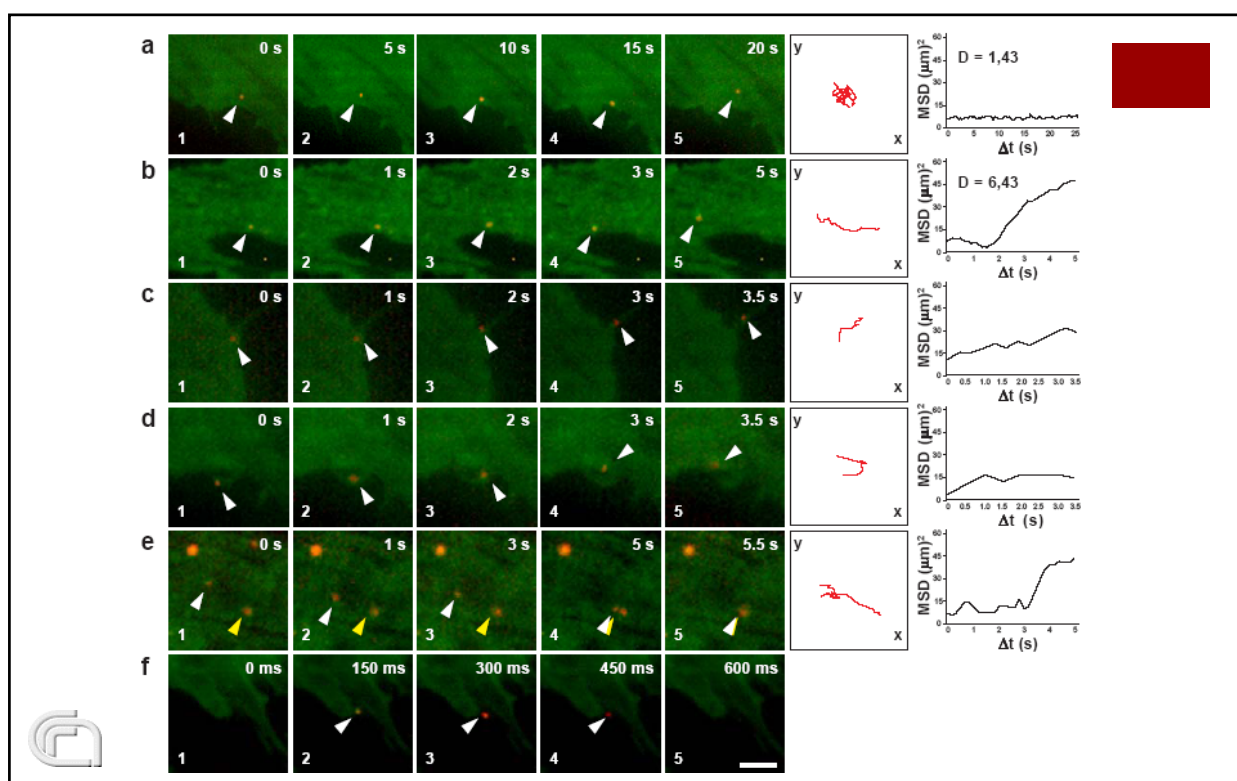
Qdots 800



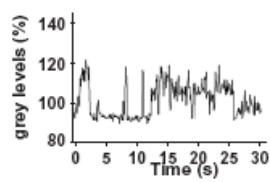
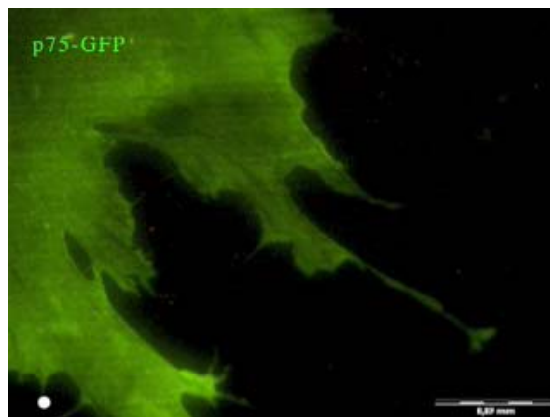
Immunocomplex

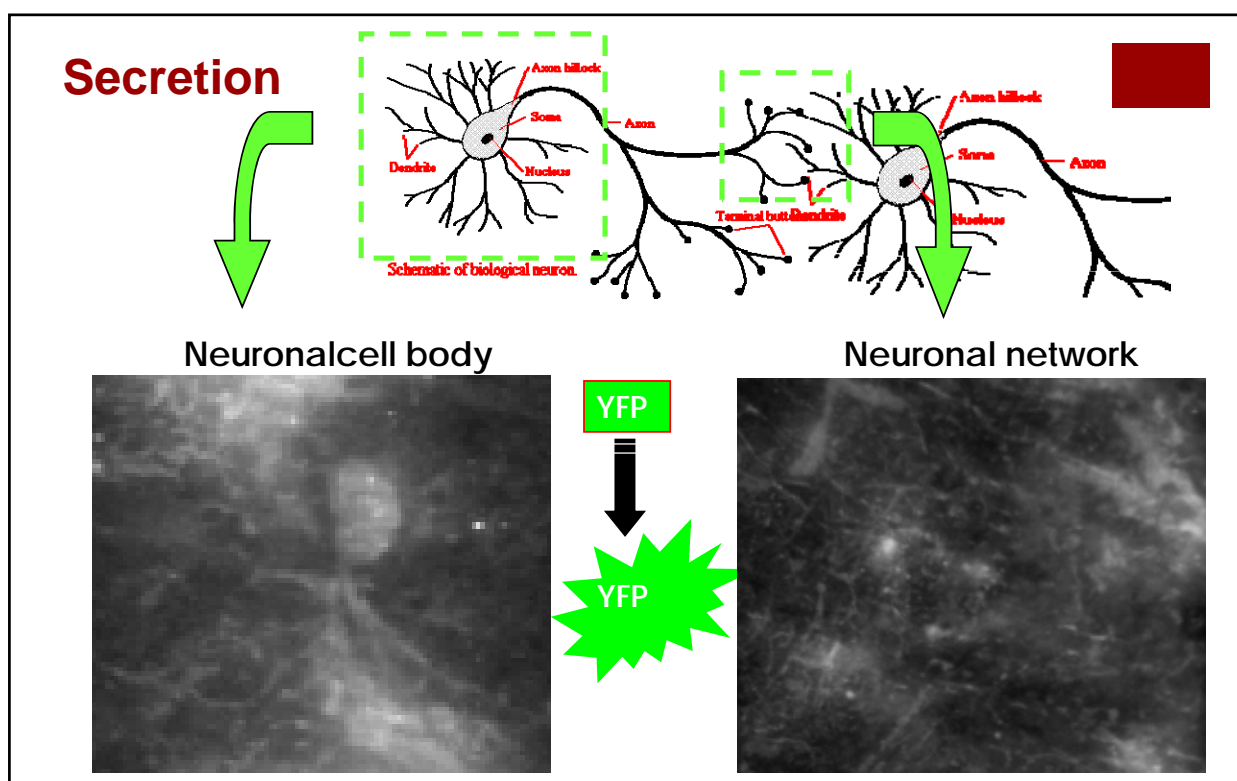
BDNF

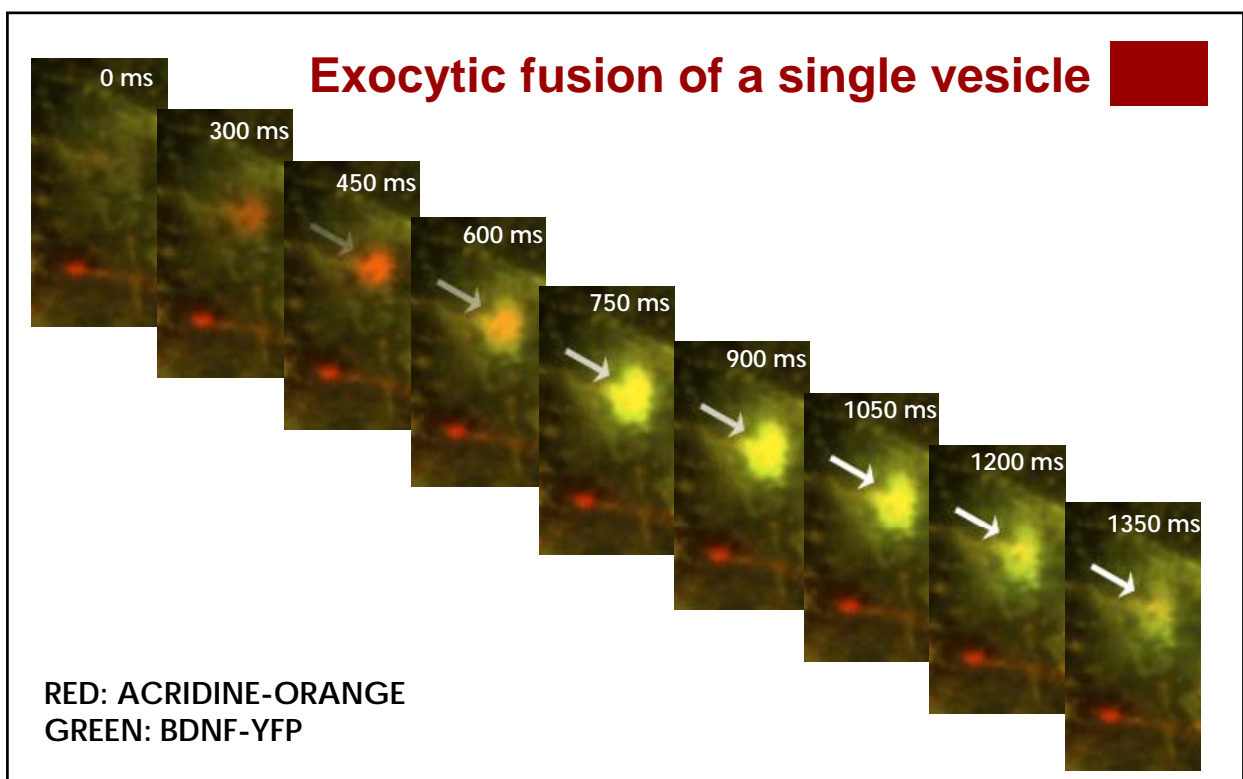




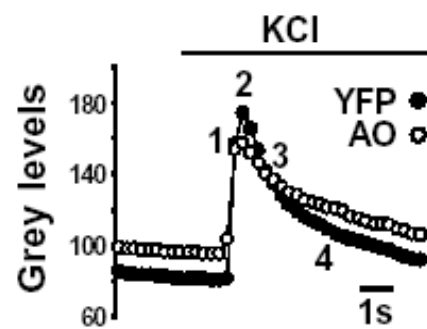
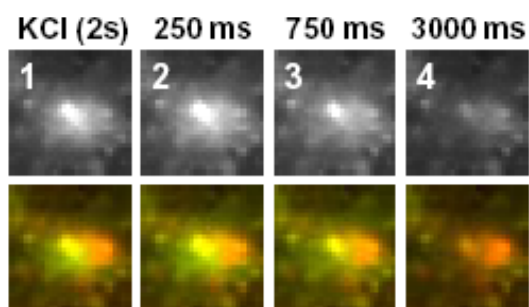
Single molecule detection



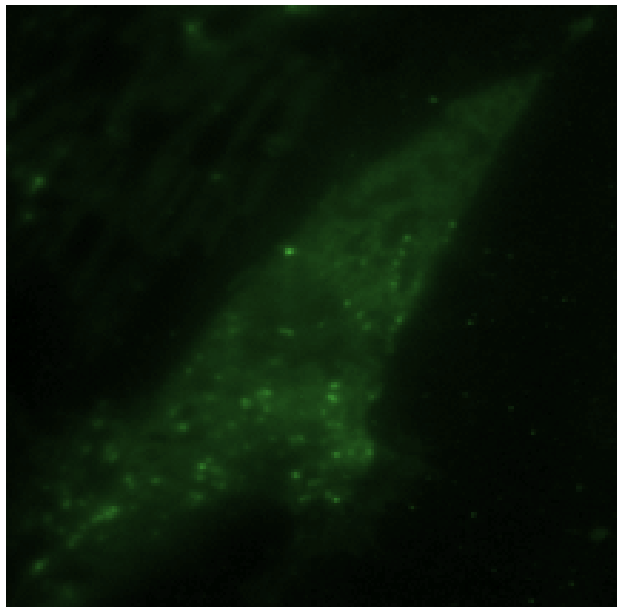




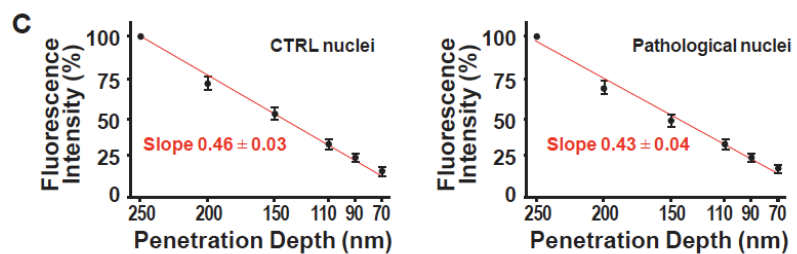
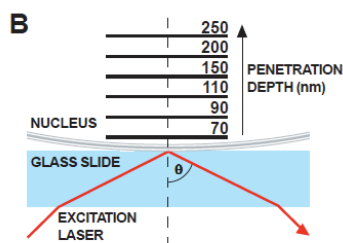
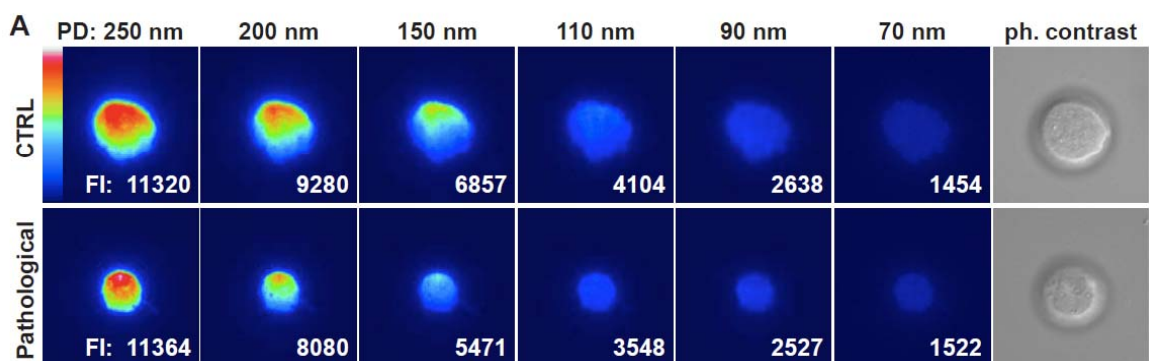
Single vesicle analysis



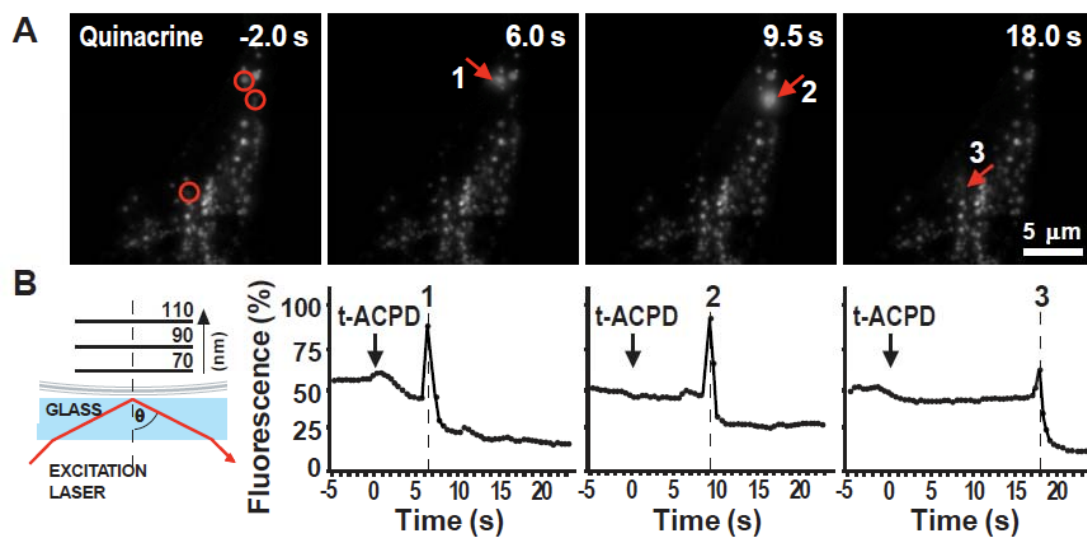
Exocytosis



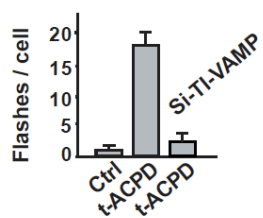
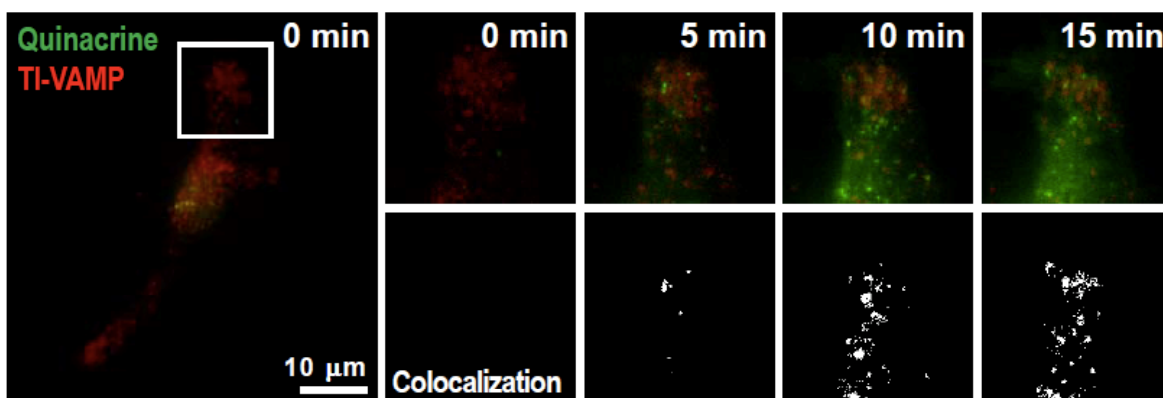
Nuclear detection



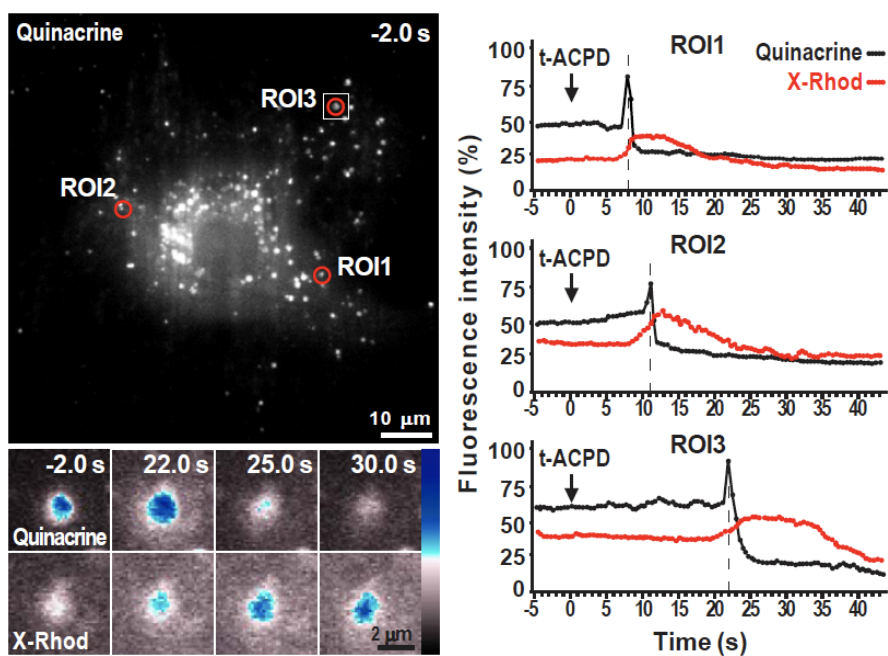
Quinacrine detection



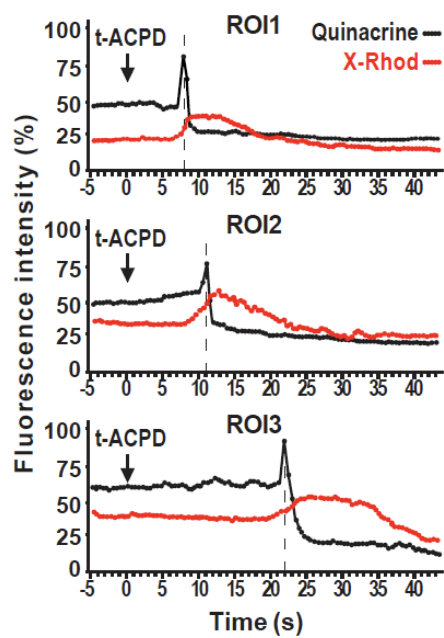
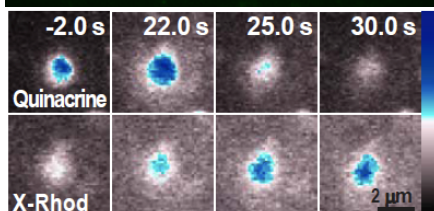
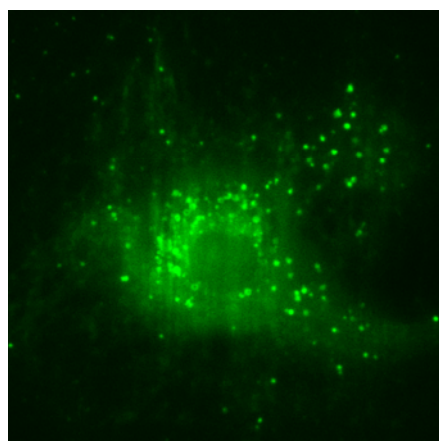
Quinacrine detection



Quinacrine and calcium detection

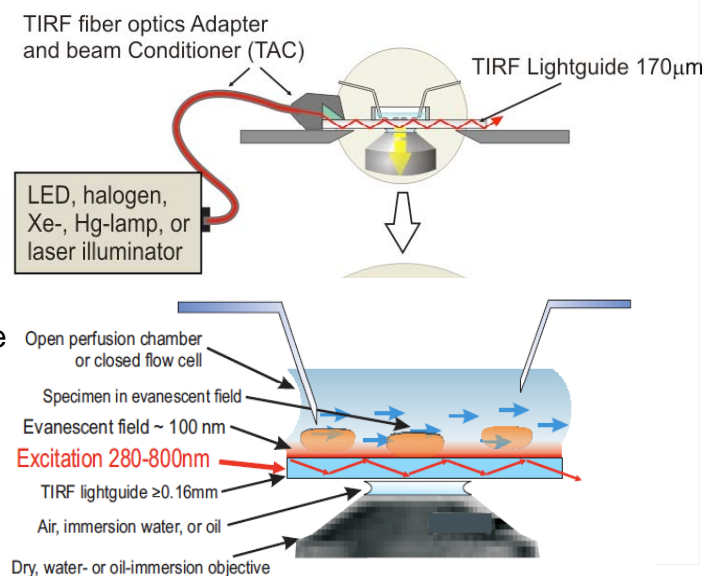


Quinacrine and calcium detection



Lightguide TIRF

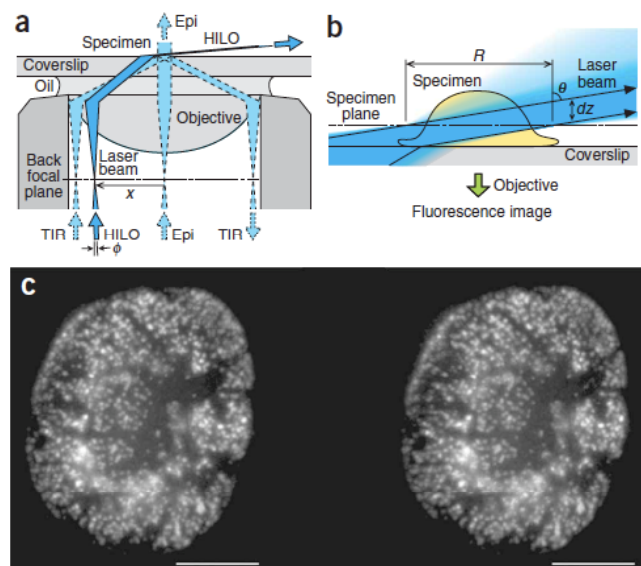
The fiber optics conducts light from the illuminator and couples the beam into TIRF lightguide. TIRF lightguide is a rectangular coverslip 22 x 40 x 0.15-0.17 mm. After entering the TIRF lightguide, the excitation beam reflects multiple times from its top and bottom surfaces and generates wide-area evanescent wave at the center of 22 x 40 mm coverslip. Sizes of the evanescent wave area are approximately 10 x 10 mm. The excitation light escapes from the opposite side of the lightguide and does not interfere with the emission channel.



HILO microscopy

The main technical challenge of single-molecule fluorescence imaging is increasing the signal/background ratio. We achieved notable success in this by inclining the illumination beam and by minimizing the illumination area.

In HILO microscopy, this thin optical sheet is used for illumination. Its thickness dz along the z -direction is roughly $dz \approx 1/4 R/\tan\theta$, which is the thickness of the geometrical optics not including the divergence, where R is the diameter of the illuminated area at the specimen plane, and θ is the incidence angle at the specimen.



Tokunaga M et al. Nat Meth. 2006

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Bergami M, 1 Santi S, Formaggio E, 4 Cagnoli C, 5 Verderio C, Blum R, Berninger B, Matteoli M, and Canossa M. Uptake and recycling of pro-BDNF for transmitter-induced secretion by cortical astrocytes. *J Cell Biol.* Oct 20;183(2):213-21.

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Choi CK, Vicente-Manzanares M, Zareno J, Whitmore LA, Mogilner A and Horwitz AR. Actin and alpha-actinin orchestrate the assembly and maturation of nascent adhesions in a myosin II motor-independent manner. *Nat Cell Biol.* 2008 Sep;10(9):1039-50.