TOTAL INTERNAL REFLECTION FLUORESCENCE MICROSCOPY TIRFM

principles & applications

LAMELIS 2019

Lasers in Medicine and Life Sciences

Advanced summer school and workshop
for students of medicine and physics
4th – 12th July 2019
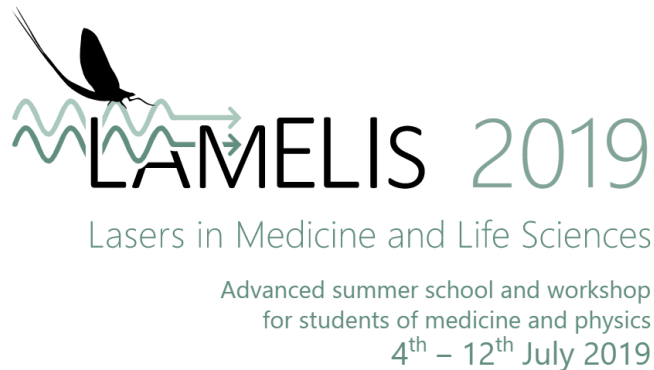
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University of Pécs – Medical School
Department of Biophysics

OVERVIEW

INTRODUCTION
- Classic and advanced resolution in the fluorescence microscope

**TOTAL INTERNAL REFLECTION FLUORESCENCE MICROSCOPY**
- Historical perspectives
- Physical principles
- How to set up a TIRF microscope?
- What TIRFM can/cannot do?
- Troubleshooting

APPLICATIONS
- Cell biology applications
- Protein biochemistry applications

Classic - resolution limited - fluorescence microscopy

- The resolution of the light microscope – how small things can be discerned – is intrinsically limited by the wave nature of light.
- Resolution/diffraction limit (19th centrury: Ernst Abbe, Lord Rayleigh)
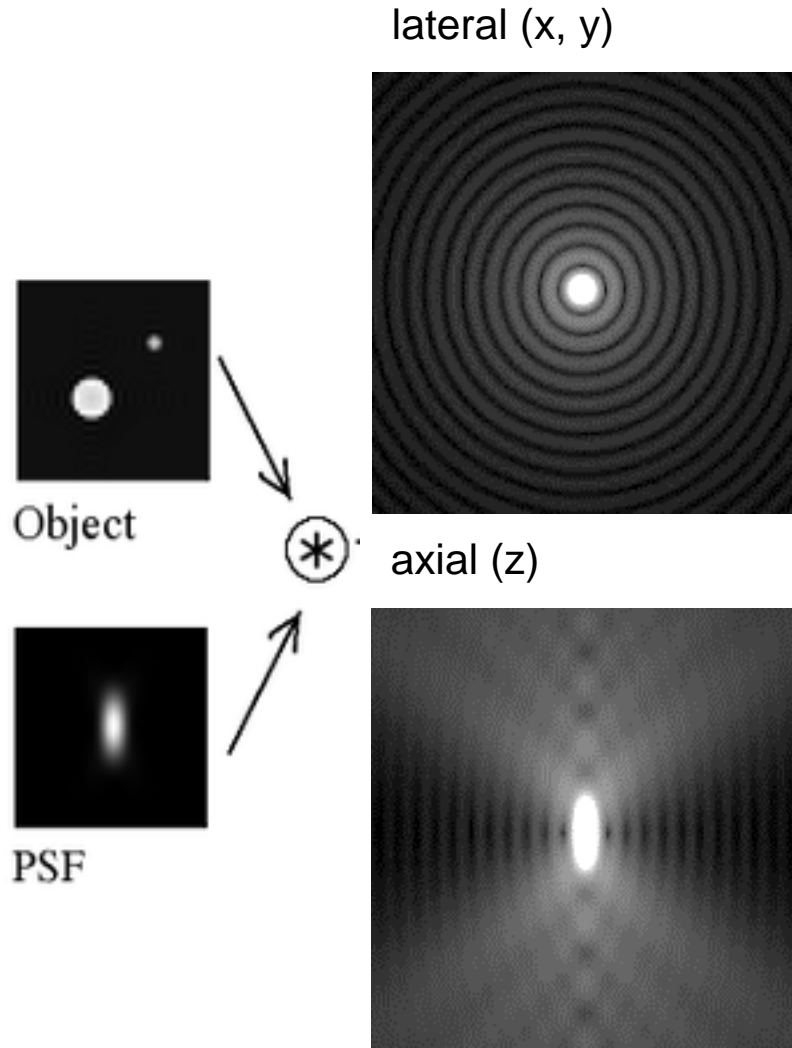
lateral (x, y)

object (ideal point) → image (3D structure)

$$image = object \otimes PSF + noise$$

PSF = point spread function ~ distortion

Object

axial (z)

PSF

$$object \sim \lambda\,(400\ -800\ nm)$$

Lateral resolution limit

$$d_{xy}= 0.61\,\frac{\lambda}{NA} \sim \mathbf{200\ nm}$$

Axial resolution limit
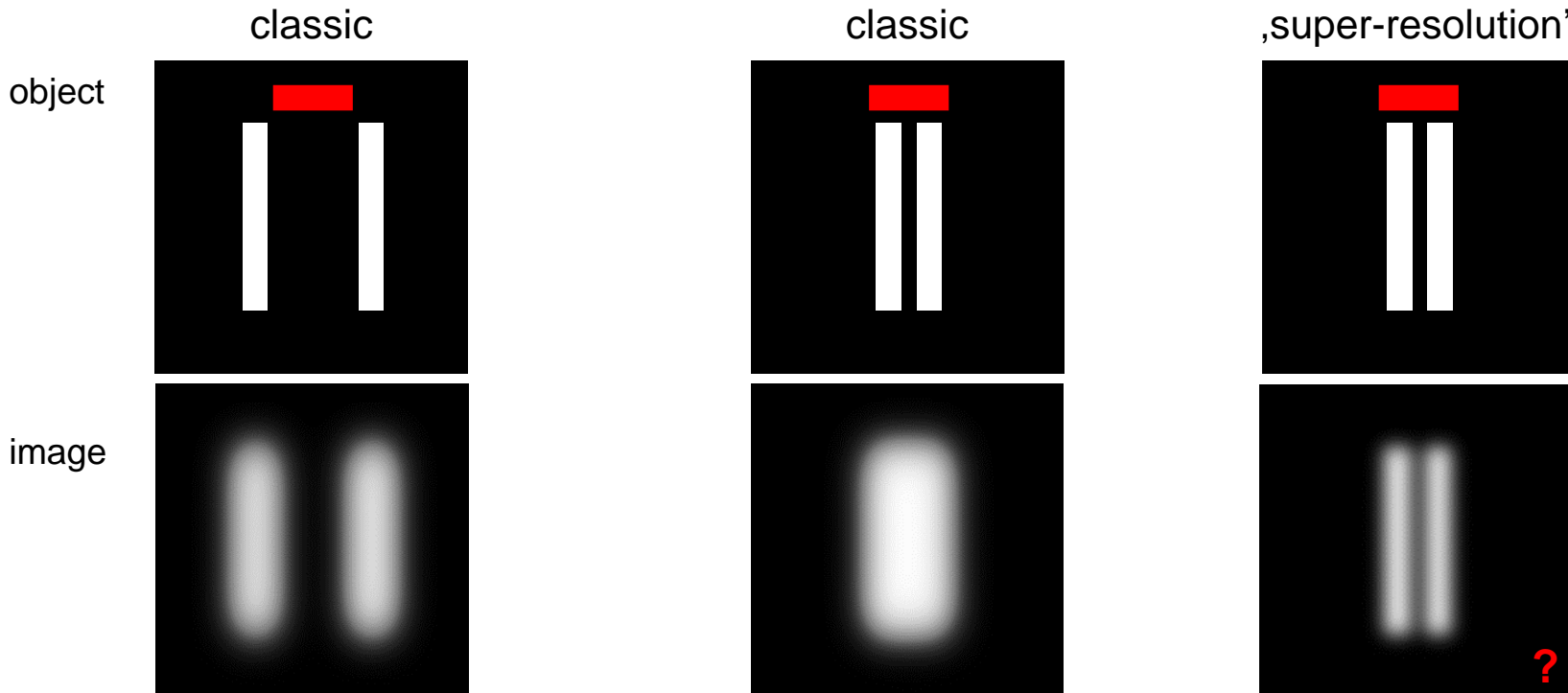
$$d_z = 2n\,\frac{\lambda}{(NA)^2} \sim \mathbf{800\ nm}$$

$$NA = n \times sin\alpha$$
numerical aperture

Classic vs super-resolution fluorescence microscopy

Limit of resolution: $d_{xy} \sim 200 \, nm$ ▬

classic	classic	,super-resolution'

object

image

Classic fluorescence microscopy cannot achive a resolution better than that is defined by the diffraction limit.

Super-resolution microscopy by surpassing the diffraction limit can provide better resolution than that is defined by the diffraction limit.

Classic vs super-resolution fluorescence microscopy

1873 diffraction limit
Ernst Abbe

$$d = \frac{\lambda}{2n\sin\alpha}$$

2014 Nobel Prize in Chemistry
"for the development of super-resolved fluorescence microscopy"

Stefan Hell   Eric Betzig   William Moerner

*Around 2000 **Eric Betzig and William E. Moerner** helped create a method in which fluorescence in **individual molecules is steered by light**. An image of very high resolution is achieved by **combining images in which different molecules are activated**.*

*In 1994, **Stefan W. Hell** developed a method in which one **light pulse** causes fluorescent molecules to glow, while another **causes all molecules except those in a very narrow area to become dark**. An image is created by sweeping light along the sample.*

http://www.nobelprize.org/nobel_prizes/chemistry/laureates/2014/

FLUORESCENCE MICROSCOPY (1911) – diffraction limited

CONFOCAL MICROSCOPY (1961)

- conjugate planes – filtering out-of-focus emission (**~ 500 – 600 nm**)
  Minsky, M. Microscopy Apparatus. US Patent 3,013,467 (1961)

SUPER-RESOLUTION FLUORESCENCE MICROSCOPY

TOTAL INTERNAL REFLECTION FLUORESCENCE MICROSCOPY – TIRFM (1981)

- evanescent wave illumination – eliminate background fluorescence (**~ < 100 nm**)
  Axelrod, D. Cell-substrate contacts illuminated by total internal reflection fluorescence. *J. Cell Biol.* 89, 141–145 (1981)

STIMULATED EMISSION DEPLETION MICROSCOPY – STED (2000)

- stimulated emission of fluorescence – PSF engineering (**~ tens of nm**)
  Klar, T. A., Jakobs, S., Dyba, M., Egner, A., **Hell, Stefan.** W. Fluorescence microscopy with diffraction resolution barrier broken by stimulated emission. *Proc. Natl Acad. Sci. USA* **97**, 8206–8210 (2000)

STRUCTURED ILLUMINATION MICROSCOPY – SIM (2000)

- spatially structured illumination/frequency mixing – extend frequency region (**~ 100 nm**)
  Gustafsson, M. G. L., D. A. Agard, and J. W. Sedat. 2000. Doubling the lateral resolution of wide-field fluorescence microscopy using structured illumination. *Proc. SPIE.* 3919:141–150.
  Gustafsson, M. G. L. 2000. Surpassing the lateral resolution limit by a factor of two using structured illumination microscopy. *J. Microsc.* 198:82–87.

SINGLE MOLECULE LOCALIZATION MICROSCOPY – STORM, PALM (2006)

- phototransformable fluorophores – Gaussian fit of PSF (**~ tens of nm**)
  Betzig, Eric. *et al.* Imaging intracellular fluorescent proteins at nanometer resolution. *Science* **313**, 1642–1645 (2006)
  Rust, M. J., Bates, M. & Zhuang, X. Sub-diffractionlimit imaging by stochastic optical reconstruction microscopy (STORM). *Nature Methods* **3**, 793–796 (2006)
  Dickson RM, Cubitt AB, Tsien RY and **Moerner William** (1997) On/off blinking and switching behaviour of single molecules of green fluorescent protein. *Nature* 388:355-358.
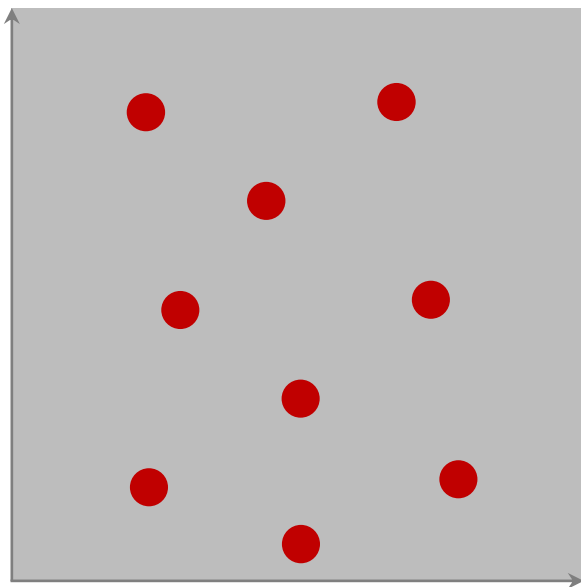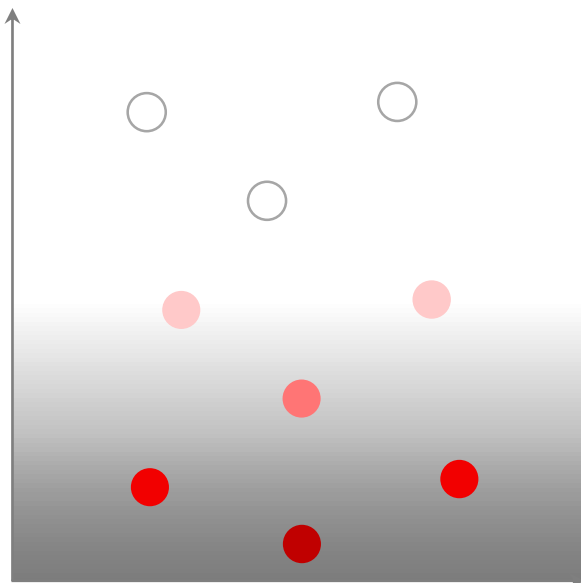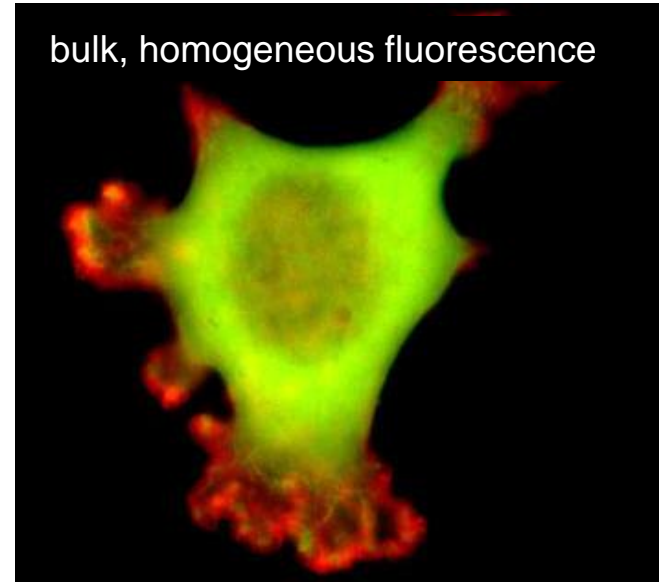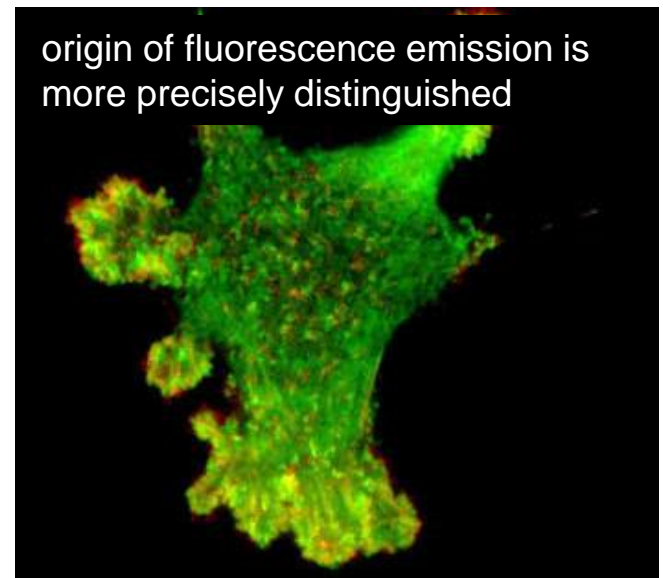
WIDE-FIELD (WF)
illuminates the entire thickness **~ μm**

bulk, homogeneous fluorescence

TIRF
selectively illuminates the most superficial layer **~ 100 nm**

origin of fluorescence emission is more precisely distinguished

B16/F1 mouse skin melanoma cell
EGFP-myosin mRFP-actin

E. J. Ambrose
Nature 1956

„In order to study the contacts formed between cells and solid surfaces, it is possible to make use of the **slight penetration of light waves into the less dense medium when totally internally reflected** at the glass/water interface."
SURFACE CONTACT MICROSCOPE

nonfluorescent, evanescent light scattering from cells

Daniel Axelrod
Journal of Cell Biology 1981

„The new method is an application … and **extension to fluorescence of the total internal reflection microscope illumination system introduced by Ambrose**."

Cell-Substrate Contacts Illuminated by Total Internal Reflection Fluorescence

DANIEL AXELROD
Biophysics Research Division and Department of Physics, University of Michigan, Ann Arbor, Michigan 48109

ABSTRACT A technique for exciting fluorescence exclusively from regions of contact between cultured cells and the substrate is presented. The technique utilizes the evanescent wave of a totally internally reflecting laser beam to excite only those fluorescent molecules within one light wavelength or less of the substrate surface. Demonstrations of this technique are given for two types of cell cultures: rat primary myotubes with acetylcholine receptors labeled by fluorescent α-bungarotoxin and human skin fibroblasts labeled by a fluorescent lipid probe. Total internal reflection fluorescence examination of cells appears to have promising applications, including visualization of the membrane and underlying cytoplasmic structures at cell-substrate contacts, dramatic reduction of autofluorescence from debris and thick cells, mapping of membrane topography, and visualization of reversibly bound fluorescent ligands at membrane receptors.

The regions of contact between a tissue culture cell and a solid substrate are of considerable interest in cell biology. These regions are obvious anchors for cell motility (1), loci for aggregation of specific membrane proteins (2–4), and convergence points for cytoskeletal filaments (2, 5, 6). Described here is a fluorescence microscope method for selectively visualizing specific molecules in cell-substrate contact regions while avoiding fluorescence excitation of the cell interior liquid medium and cellular debris. Other potential applications of this method include viewing fluorescence-marked receptors at very low cell surface concentrations, cytoplasmic filaments in thick cells, and fluorescent agonists that bind reversibly to the cell membrane.

The new method is an application of total internal reflection fluorescence (TIRF) to cellular microscopy and is an extension to fluorescence of the total internal reflection microscope illumination system introduced by Ambrose (7) to detect light scattered at cell-substrate contacts. TIRF microscopy utilizes a light beam in the substrate that is obliquely incident upon the substrate liquid interface at an angle greater than the critical angle of refraction. At this angle, the light beam is totally reflected by the interface. However, an electromagnetic field called the "evanescent wave" does penetrate into the liquid medium. The evanescent wave propagates parallel to the surface with an intensity I that decays exponentially with perpendicular distance z from the surface:

$$I = I_o \exp(-z/d) \qquad (1)$$

The characteristic exponential decay depth d is:

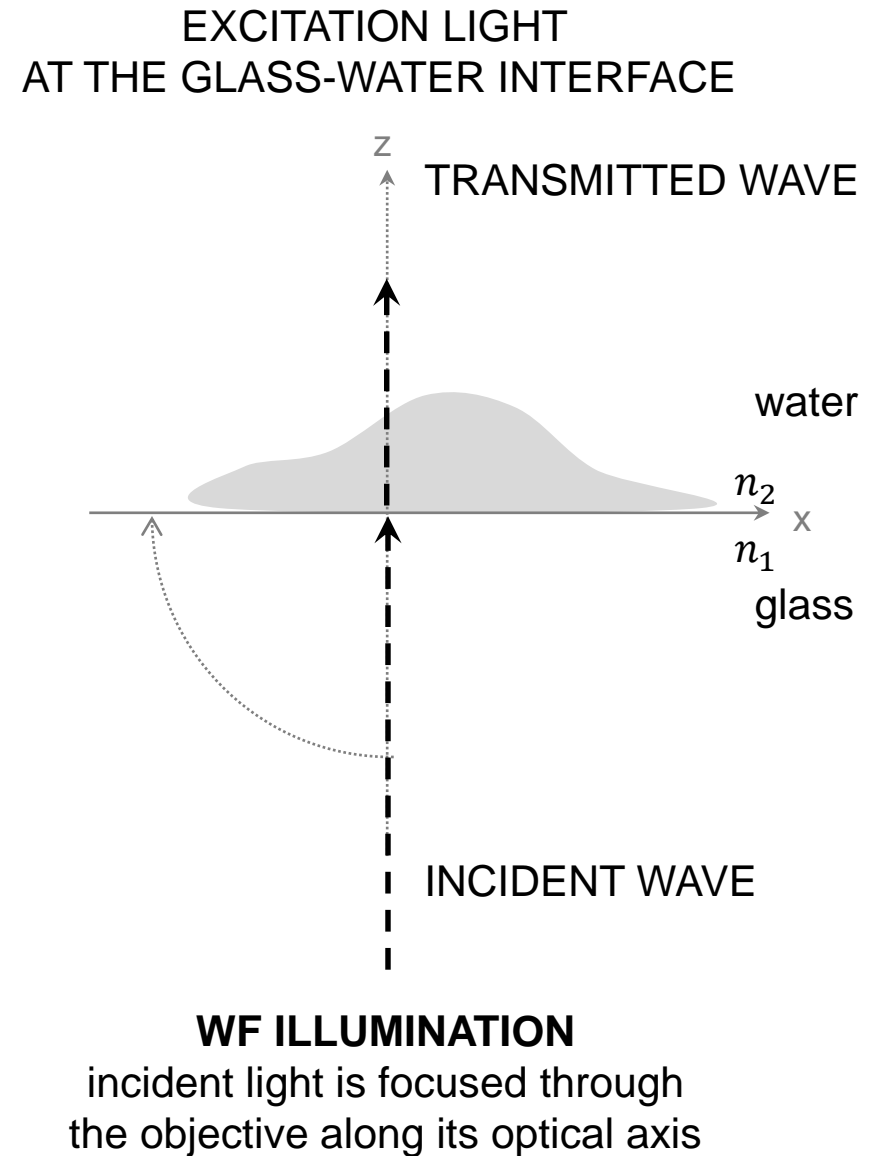$$d = \frac{\lambda}{4\pi n_2}\left(\frac{\sin^2\theta}{\sin^2\theta_c} - 1\right)^{-1/2}, \qquad (2)$$

where n_1 = refractive index of the substrate; n_2 = refractive index of the liquid medium; θ_c = the critical angle of incidence = $\sin^{-1} n_2/n_1$; θ = the angle of incidence, $\theta > \theta_c$; and λ = the wavelength of incident light in vacuum. The decay depth d decreases with increasing θ. Except for θ close to θ_c (where $d \rightarrow \infty$), d is on the order of λ or smaller. I_o, the intensity of the evanescent wave at $z = 0$, is on the order of the incident light intensity except for angles of incidence very near the critical angle (8). Therefore, for most experimental configurations, a fluorescent molecule located in the evanescent wave at $z = 0$ will be excited with roughly the same efficiency as it would if it were located in the incident beam.

A fluorescent molecule located close to the surface in the evanescent wave can become excited and emit fluorescence; molecules much farther away will not be excited. The efficiency of excitation decays exponentially according to Eqs. 1 and 2. For typical experiments described here, identical fluorescent molecules located at 1, 10, 100, and 1,000 nm from the surface will emit relative fluorescence intensities of 0.99, 0.92, 0.43, and 0.0002, respectively. For cells adhering to the surface, only fluorescent molecules at or near the cell surface in the regions of closest contact with the substrate will be excited significantly.

TIRF has been employed previously to study surface interactions in a variety of molecular systems, including solutions of fluorescein (9) and serum albumin (10, 11) at glass surfaces, and antibodies at antigen coated surfaces (12). More recently, TIRF has been combined with fluorescence photobleaching recovery and fluorescence correlation spectroscopy to study the surface adsorption/desorption kinetics of fluorescent macromolecules (13, 14) and viruses (15).

A completely unrelated transmitted illumination technique,

EXCITATION LIGHT
AT THE GLASS-WATER INTERFACE

z

TRANSMITTED WAVE

water

n_2

x

n_1

glass

INCIDENT WAVE

WF ILLUMINATION
incident light is focused through
the objective along its optical axis

Total internal reflection – geometrical optical principles

SNELL'S LAW OF REFRACTION

$$n_1 sin\alpha_1 = n_2 sin\alpha_2$$

glass ($n_1 = 1.515$) **>** water ($n_2 = 1.333$) interface
angle of incidence (α_1) **<** angle of refraction (α_2)

CRITICAL ANGLE

angle of refraction (α_2) = 90^0
angle of incidence (α_1) $\equiv \boldsymbol{\alpha_{critical}}$

$$\boldsymbol{\frac{n_1}{n_2} sin\alpha_{critical} = 1}$$

$$sin\alpha_{critical} = \frac{n_2}{n_1} = \frac{1.333}{1.515} = 0.88$$

$$\boldsymbol{\alpha_{critical} = 61.63^o}$$

EXCITATION LIGHT
AT THE GLASS-WATER INTERFACE

TRANSMITTED WAVE

SUPERCRITICAL

$\alpha_1 > \alpha_{critical}$

$\alpha_1 = \alpha_{critical}$

$\alpha_1 < \alpha_{critical}$

SUBCRITICAL

INCIDENT WAVE
oblique angle

α_2

$\alpha_2 = 90^0$ n_2
                      water
                      n_1
                      glass

α_1

TOTAL INTERNAL
REFLECTION

REFLECTED WAVE

objective optical axis

Notation:
1: incident wave/1st medium
2: transmitted wave/2nd medium
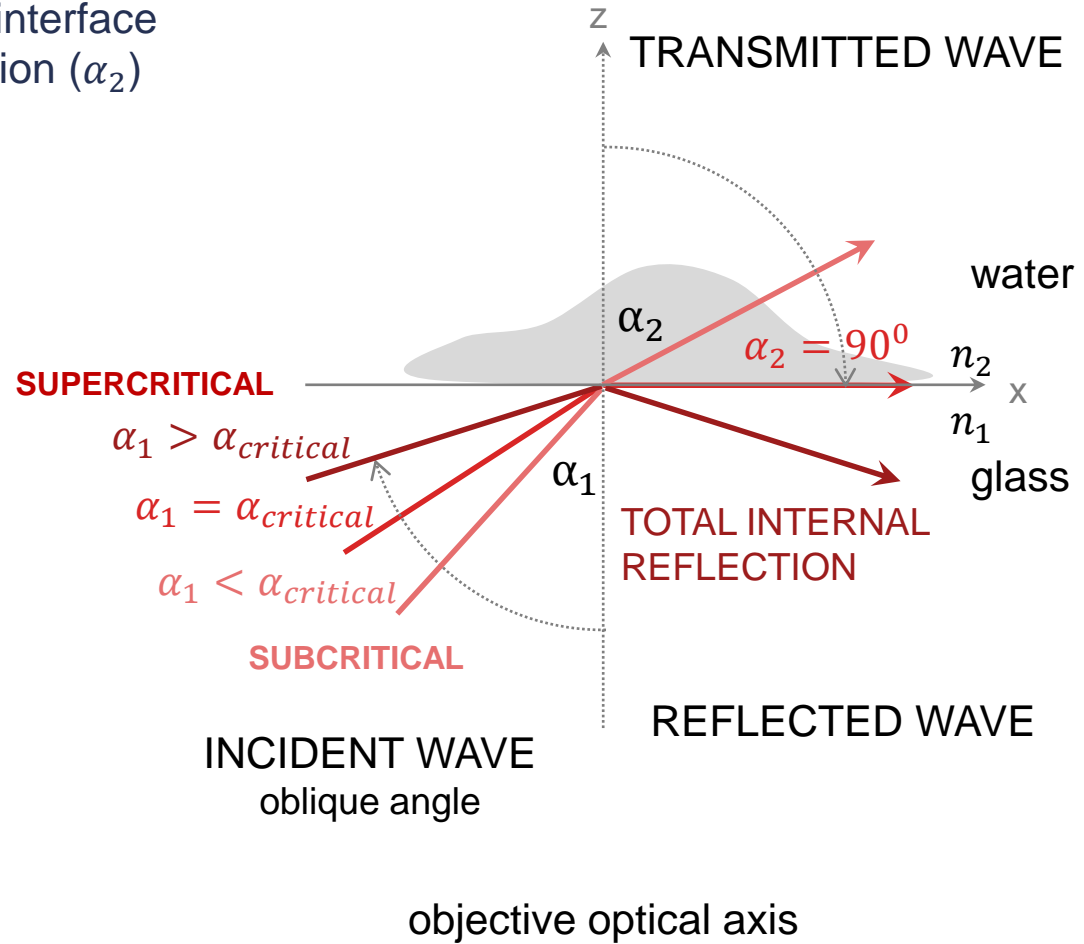
Intensity response of fluorophores as a function of α_1

single refractive index change

monochromatic plane wave

INCIDENT: $\vec{E}_1(\vec{r}, t) = \vec{E}_1 \exp\left[i(\vec{k}_1 \vec{r} - \omega t)\right]$

TRANSMITTED: $\vec{E}_2(\vec{r}, t) = \vec{E}_2 \exp\left[i(\vec{k}_2 \vec{r} - \omega t)\right] =$

$= \vec{E}_2 \exp\left[i(k_2 \sin\alpha_2 x + k_2 \cos\alpha_2 z - \omega t)\right]$

$ik_2(\sin\alpha_2 x + \cos\alpha_2 z) = ik_2\left(\dfrac{\sin\alpha_1}{n} x + i\sqrt{\dfrac{\sin^2\alpha_1}{n^2} - 1}\, z\right)$

CRITICAL: $\alpha_1 = \alpha_c$

$\vec{E}_2(\vec{r}, t) = \vec{E}_2 exp[i(k_2 x - \omega t)]$

SUPERCRITICAL: $\alpha_1 > \alpha_c$

$\vec{E}_2(\vec{r}, t) =$

$= \vec{E}_2\, exp\left[i\left(k_2 \dfrac{\sin\alpha_1}{n} x - \omega t\right)\right] \cdot \boxed{exp\left[-k_2 \sqrt{\dfrac{\sin^2\alpha_1}{n^2} - 1}\, z\right]}$

z

TRANSMITTED WAVE

$\vec{E}_2(\vec{r}, t)$

$\vec{k}_2$

water

α_2

n_2

X

n_1

glass

α_1

$\vec{k}_1$

$\vec{E}_1(\vec{r}, t)$

INCIDENT WAVE
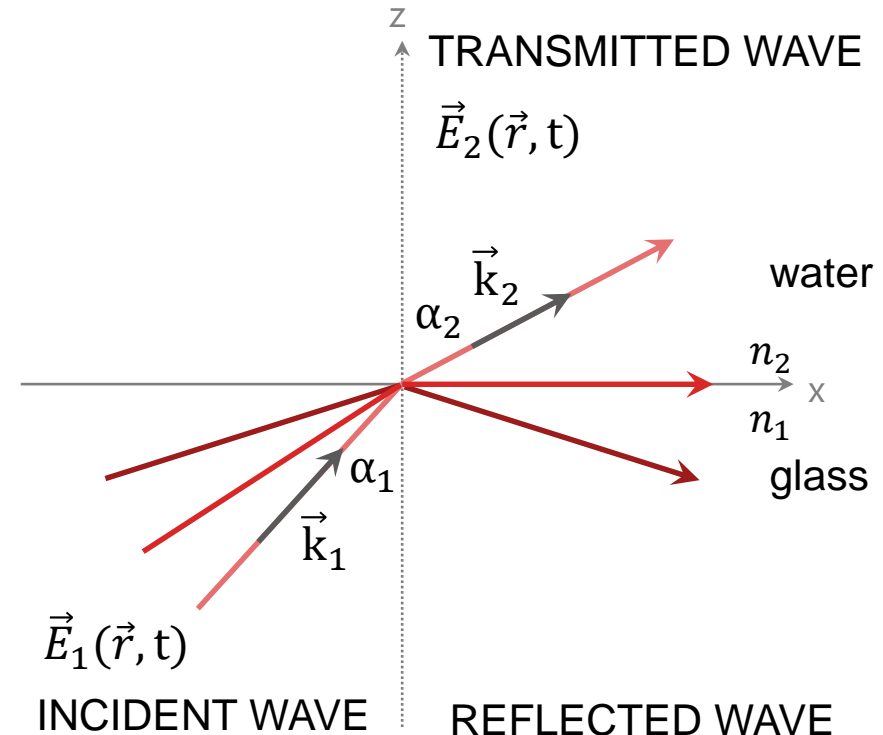
REFLECTED WAVE

Upon TIR an EM field appears in the second medium right above the surface. The transmitted wave

- **propagates perpendicular to the surface (z direction)**
- **attenuated exponentially = EVANESCENT ('disappear in space')**

EVANESCENT WAVE

$$\vec{E}_{\text{evanescent}}(\vec{r}, t) = \vec{E}\,exp[-k_2\sqrt{\frac{sin^2\alpha_1}{n^2} - 1}z]$$

$$= \vec{E}\,exp\left[-\frac{2\pi}{\lambda}\frac{1}{n_2}\sqrt{sin^2\alpha_1 n_1^2 - n_2^2}z\right]$$

INTENSITY PROFILE

$$I(z) = |\vec{E}|^2 = I_0\,exp\left(-\frac{z}{d}\right)$$

PENETRATION DEPTH/DECAY LENGTH

$$d = \frac{\lambda}{4\pi n_1}(sin^2\alpha_1 - (\frac{n_2}{n_1})^2)^{-\frac{1}{2}}$$

z = distance from the interface (nm)

evanescent   epi

$$n_2 = 1.333$$

$$n_1 = 1.515$$

$$I(d) = \frac{I_0}{e} \qquad \lambda_0 = 491 \text{ nm}$$

$$\alpha_{\text{incidence}} = 65°$$

d

water

glass

relative field intensity
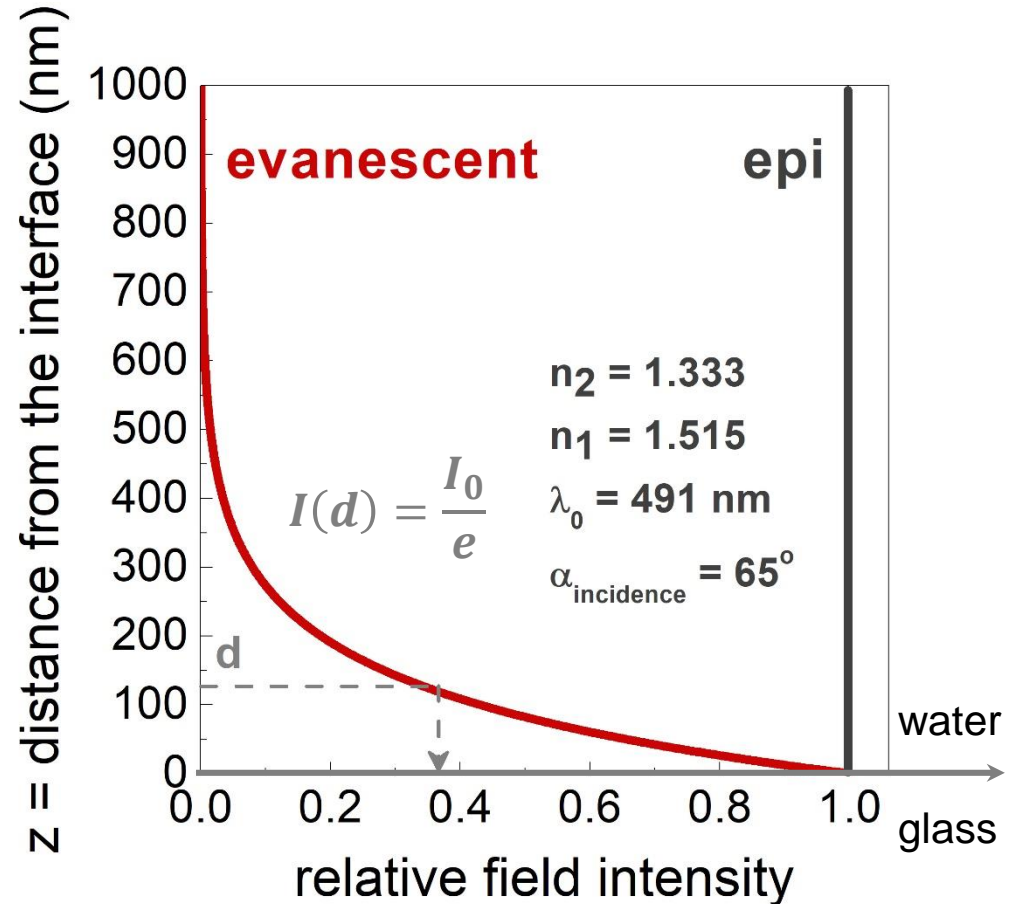
The decay length determines the axial resolution!
The shorter the decay length, the better the resolution.
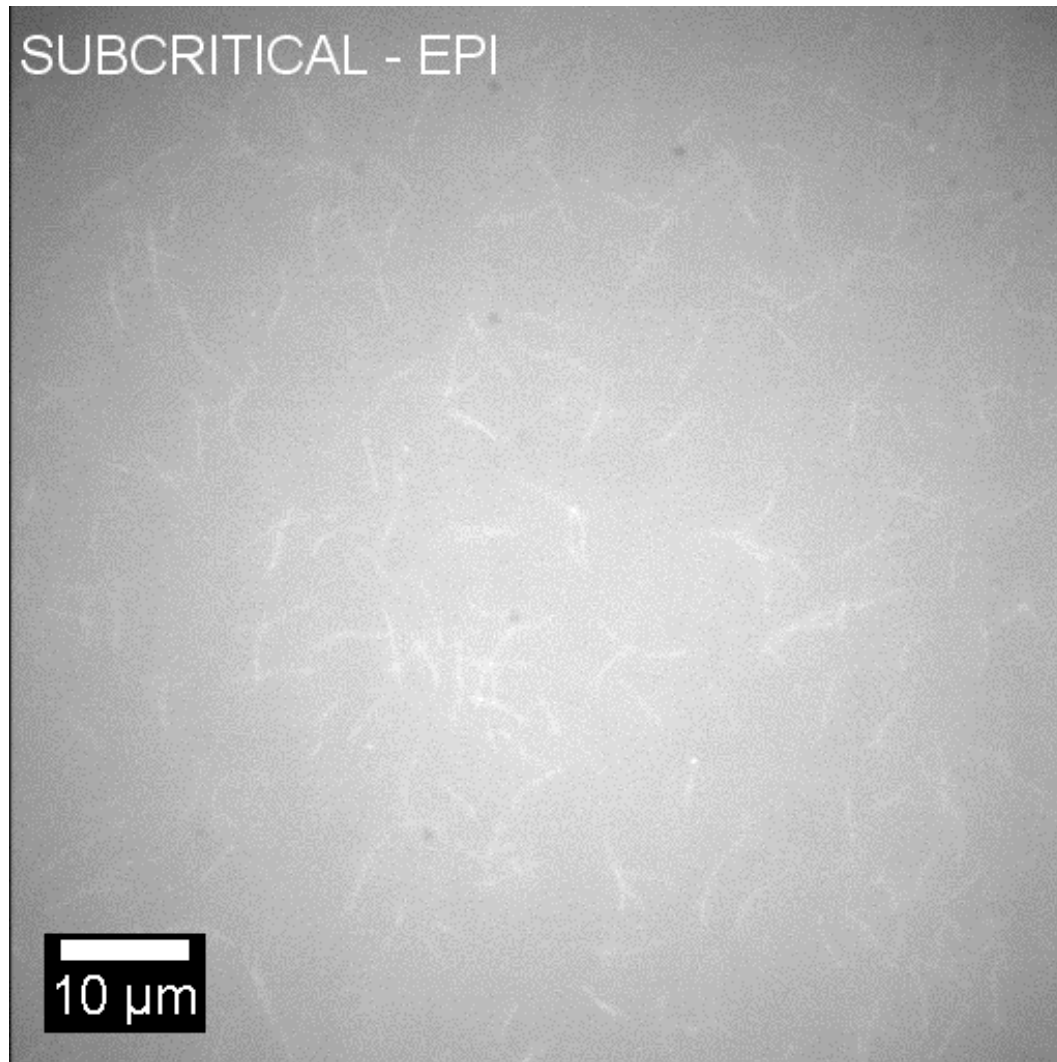
The onset of TIRF is obvious as a sudden darkening of the background and a flat 2D look to the features near the surface.

Alexa488NHS labeled actin filaments, Olympus IX81 inverted microscope, λ_{ex} = 491 nm, 60× 1.49 NA

**Wide-field
subcritical angle**
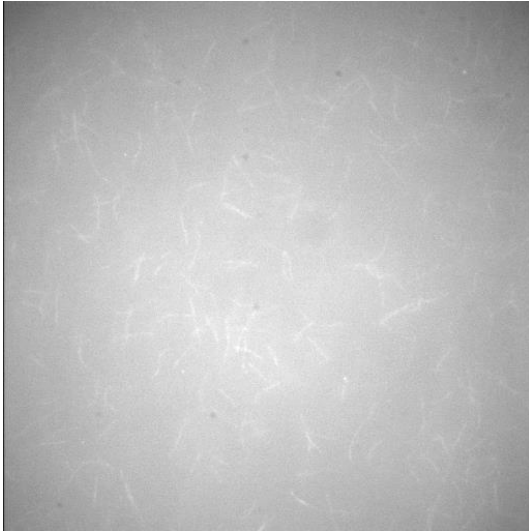
critical angle
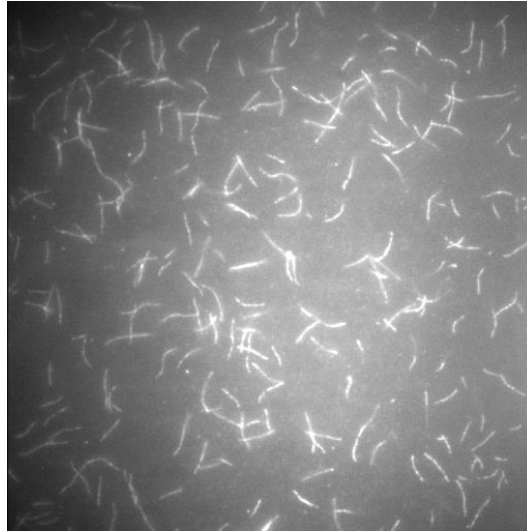
**TIRF
supercritical angle**

$$sin\alpha_{critical} = \frac{n_2}{n_1}$$

$n_2 = 1.333$

$n_1 = 1.333$

$\alpha_{critical} \sim 60 - 70^o$

$n_1 = 2.400$

refractive index ratio = n_2/n_1
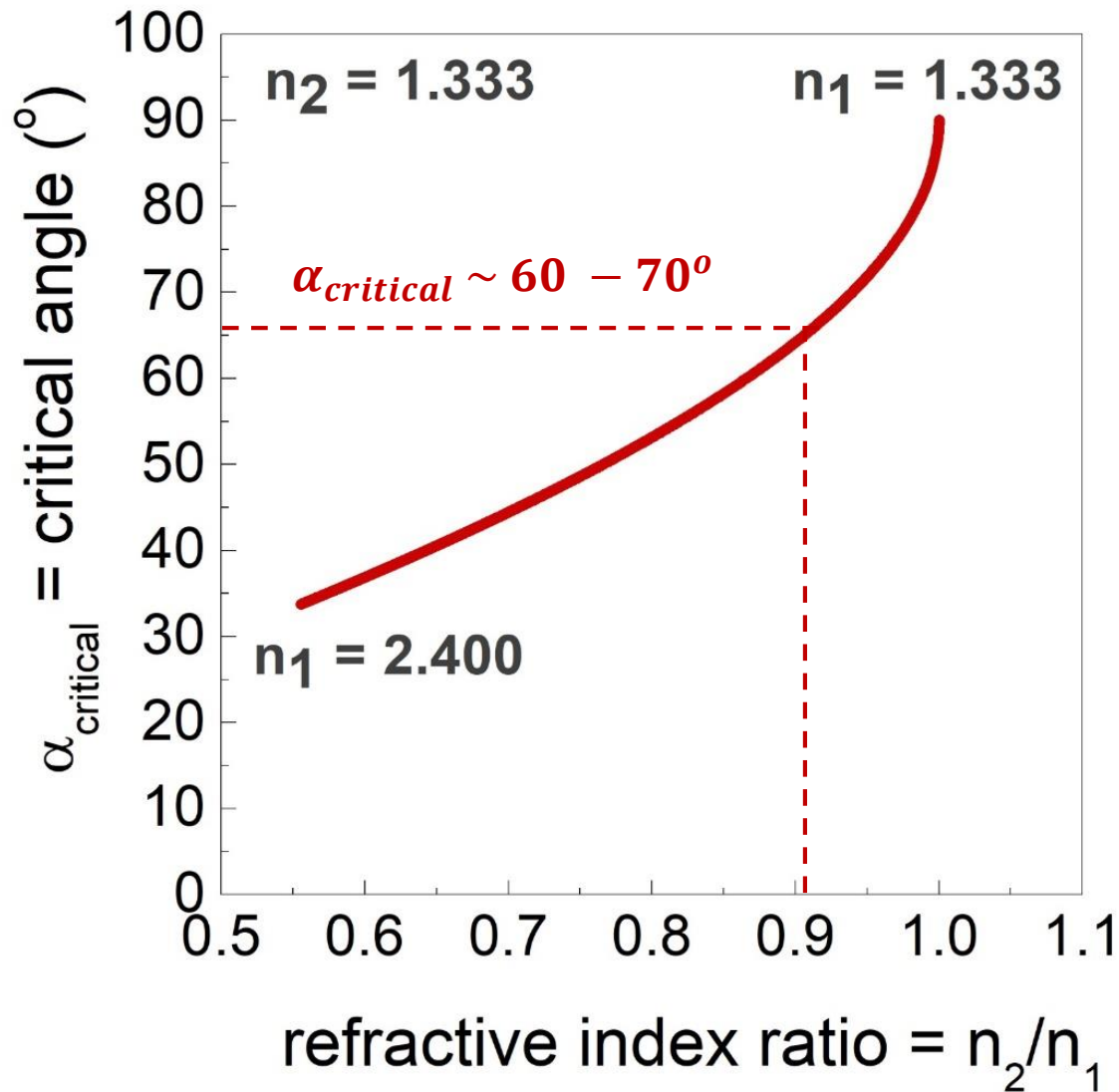
If $\frac{n_2}{n_1}$ is small, $\alpha_{critical}$ is shallow and TIR is easily achieved.

$$d = \frac{\lambda}{4\pi n_1}(sin^2\alpha_1 - (\frac{n_2}{n_1})^2)^{-\frac{1}{2}}$$

$\lambda \downarrow \rightarrow d \downarrow$

as the wavelength decreases, the depth is narrower.

$\alpha_1 \uparrow \rightarrow d \downarrow$

$\alpha_1 \rightarrow \alpha_{critical}: d \rightarrow \infty$

as the angle of incidence increases, the depth becomes narrower.
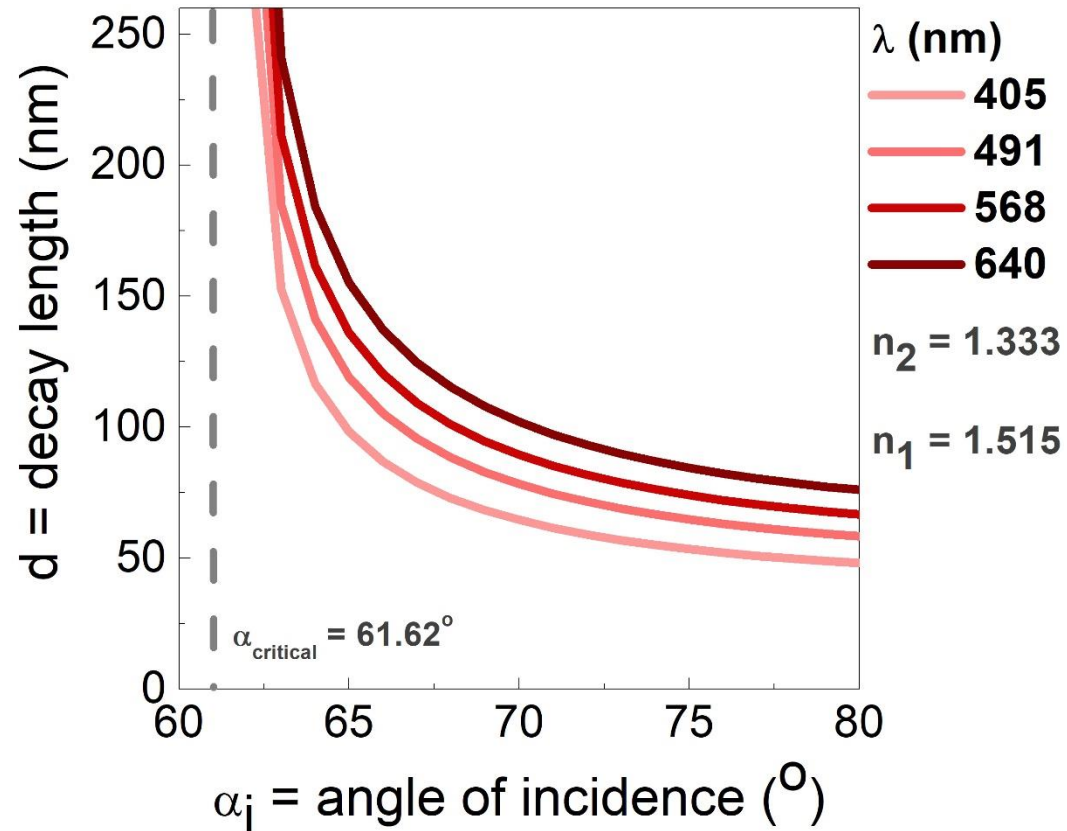
The shallower the evanescent field (smaller the d), the better the resolution.
The larger the angle of incidence, the better the resolution.

$$d = \frac{\lambda}{4\pi n_1}(sin^2\alpha_1 - (\frac{n_2}{n_1})^2)^{-\frac{1}{2}}$$

typical refractive index region of biological samples

$n_2 \uparrow \rightarrow d \uparrow$

as n_2 increases, the depth increases, which negatively influences resolution!

$\alpha_1 \uparrow \rightarrow$ sensitivity to $n_2 \downarrow$

as the angle of incidence increases, the sensitivity of the evanescent field to n_2 decreases.

less uniform – more uniform

angle of incidence
α_i (°)

— 68.86
— 70.63
— 73.57
— 78.46

$n_1 = 1.515$
$\lambda = 491$ nm

d = decay length (nm)

n_2 = refractive index of the sample
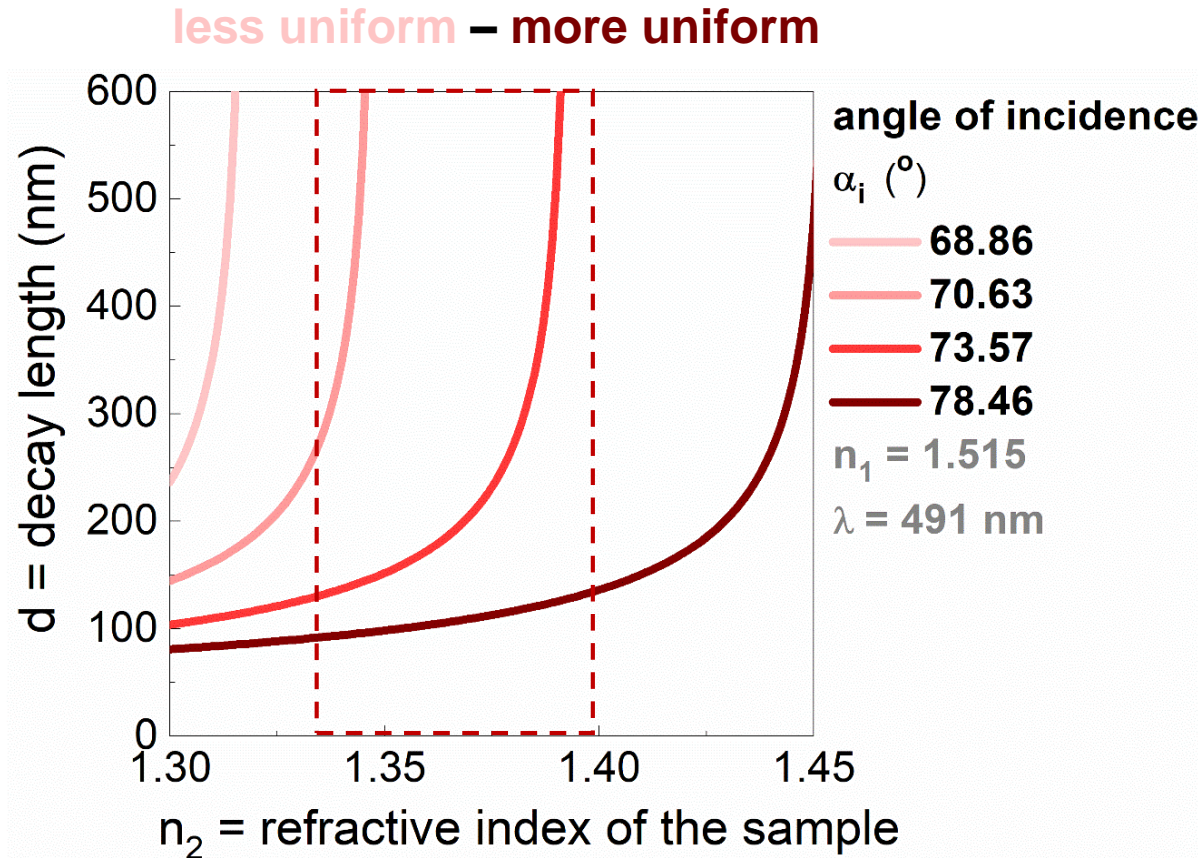
We do have refractive index inhomogeneity in the sample.
The larger the angle of incidence, the refractive index inhomogeneity of the sample has less influence on the decay length of the evanescent field, TIR illumination is more robust.
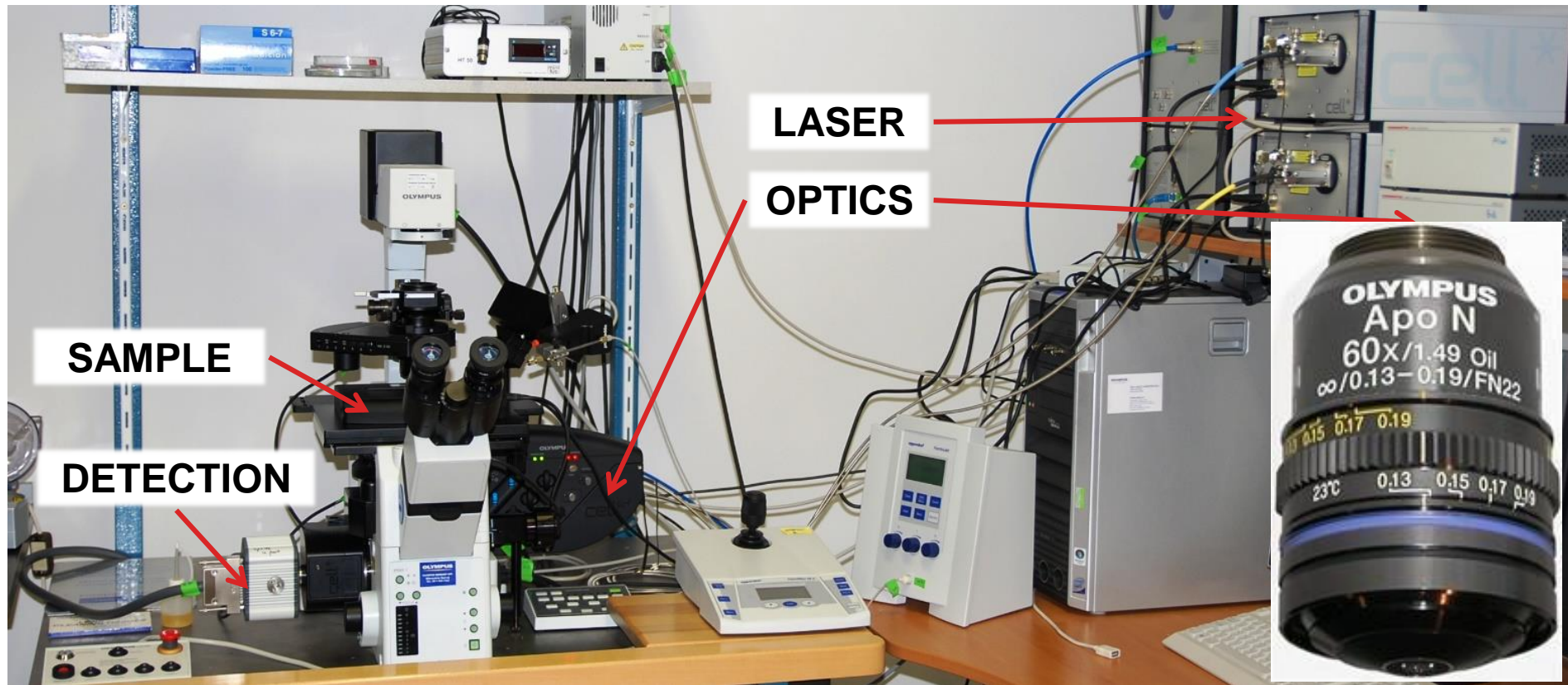
upgrade checklist:
- laser(s) for illumination
- special objective
- optics to couple, focus and adjust laser beam(s)

- **REFRACTIVE INDEX RATIO**

THE REFRACTIVE INDEX OF THE 2ND MEDIUM HAS TO BE LOWER THAN THAT OF THE 1ST ONE

$n_1 > n_2$

$n_{coverglass(1)} = 1.515$
$n_{water(2)} = 1.333 - n_{cell(2)} = 1.38$

use of immersion medium ($n_{oil} = 1.515$) to provide continuous optical contact along the light path

- **CRITICAL ANGLE OF ILLUMINATION**

Limits resolution and the robustness of TIRFM!!

THE ANGLE OF INCIDENCE HAS TO BE LARGER THAN THE CRITICAL ANGLE

$\alpha_{incidence} > \alpha_{critical}$

for glass : water interface: $\alpha_{critical} = 61.62^o$
for glass : cell interface: $\alpha_{critical} = 65.62^o$
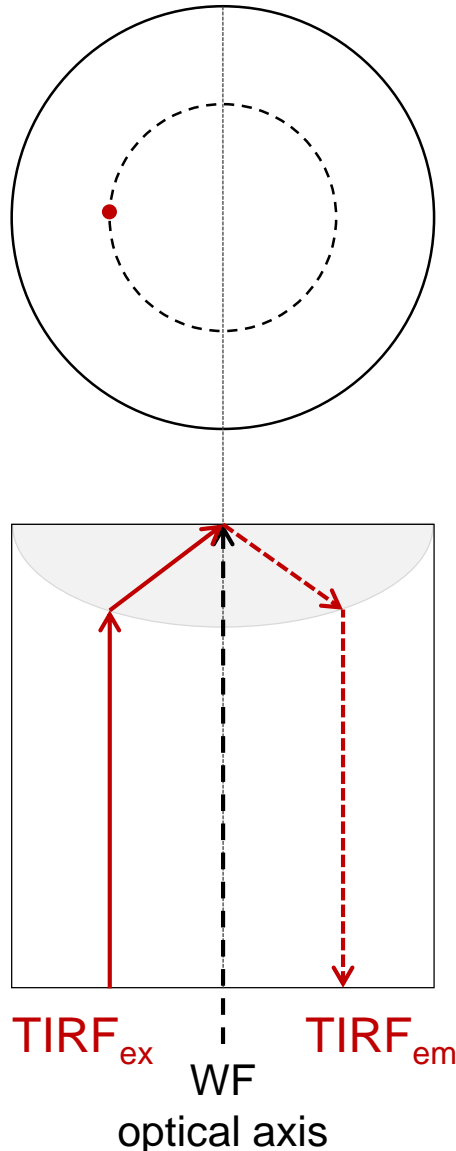
Laser beam alignment
- in wide-field mode it is ‚perfectly' aligned to the optical axis of the objective
- is focused in the back focal plane of the objective

collimated light; all the rays are parallel and all go at the same angle (all are totally internally reflected)

- in off axis position relative to the optical axis of the objective

TIRF$_{ex}$ TIRF$_{em}$

WF
optical axis

Figure 5

Specimen

Stage

Apo 100x High NA Objective

Dichromatic Mirror

Tube Lens

Collimated and Focused Laser Illumination

Cooled CCD Camera

533 Nanometer 25mW Laser

Laser Expander and Focus

Barrier Filter

glass : water interface

$n_{water\ (2)} = 1.333$

$n_{glass(1)} = 1.515$

$n_{immersion(1)} = 1.515$

$\alpha_{critical} = 61.62^o$

$NA(minimum) = 1.515 \times sin61.62^o = \mathbf{1.33}$

$NA \sim \mathbf{1.4}$

glass : cell interface

$n_{cell(2)} = 1.38$

$n_{glass(1)} = 1.515$

$n_{immersion(1)} = 1.515$

$\alpha_{critical} = 65.62^o$

$NA(minimum) = 1.515 \times sin65.63^o = \mathbf{1.38}$

$NA > \mathbf{1.4}!$

Numerical aperture of the objective

	WD (mm)	NA	n_1 immersion	n_2 cell	critical angle (º)	maximum angle (º)	utilized NA fraction
plan apochromatic	-	1.4	1.515	1.38	65.63	67.53	0.02
UAPON 150xTIRF	0.08	1.45	1.515	1.38	65.63	73.15	0.07
APON 60XTIRF UAPON 100xTIRF	0.1 0.1	1.49	1.515	1.38	65.63	79.57	0.11
APON 100XTIRF	0.08	1.65 *	1.788	1.38	50.51	67.34	0.27

* special immersion oil and glass coverslip are needed

The excitation light has to pass through the portion of the numerical aperture cone that is greater than ~ 1.4.

The larger the NA, the more flexibility you have, the more robust your TIRFM.

Being on the edge gives less flexibility.

AVAILABLE NUMERICAL APERTURE MARGIN
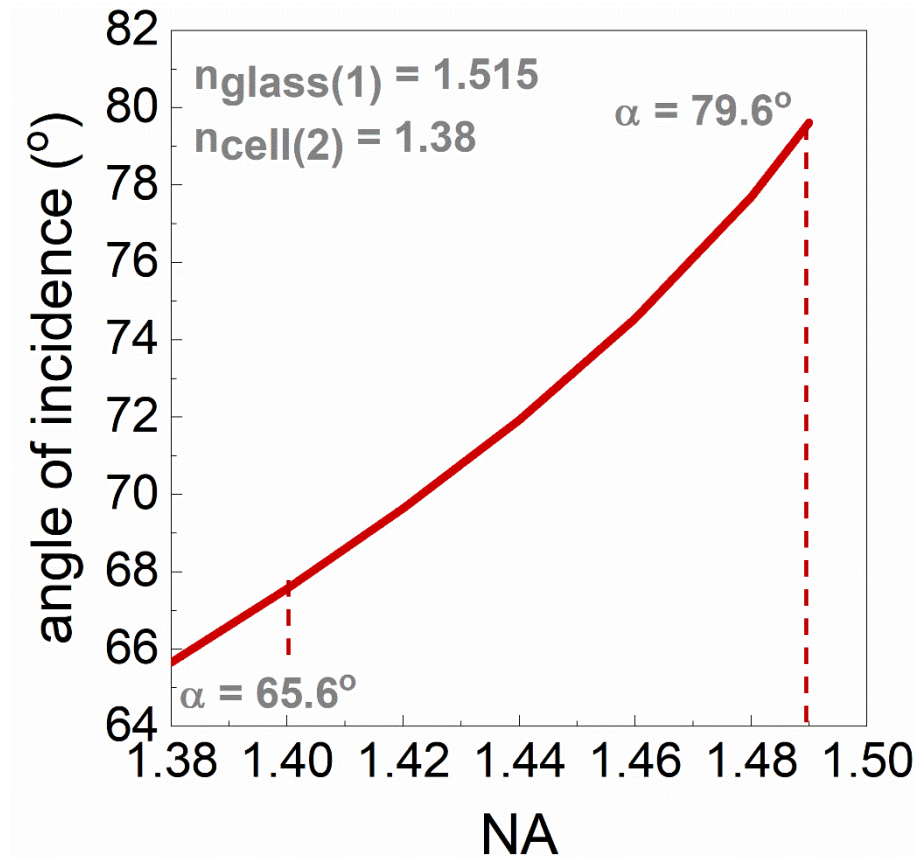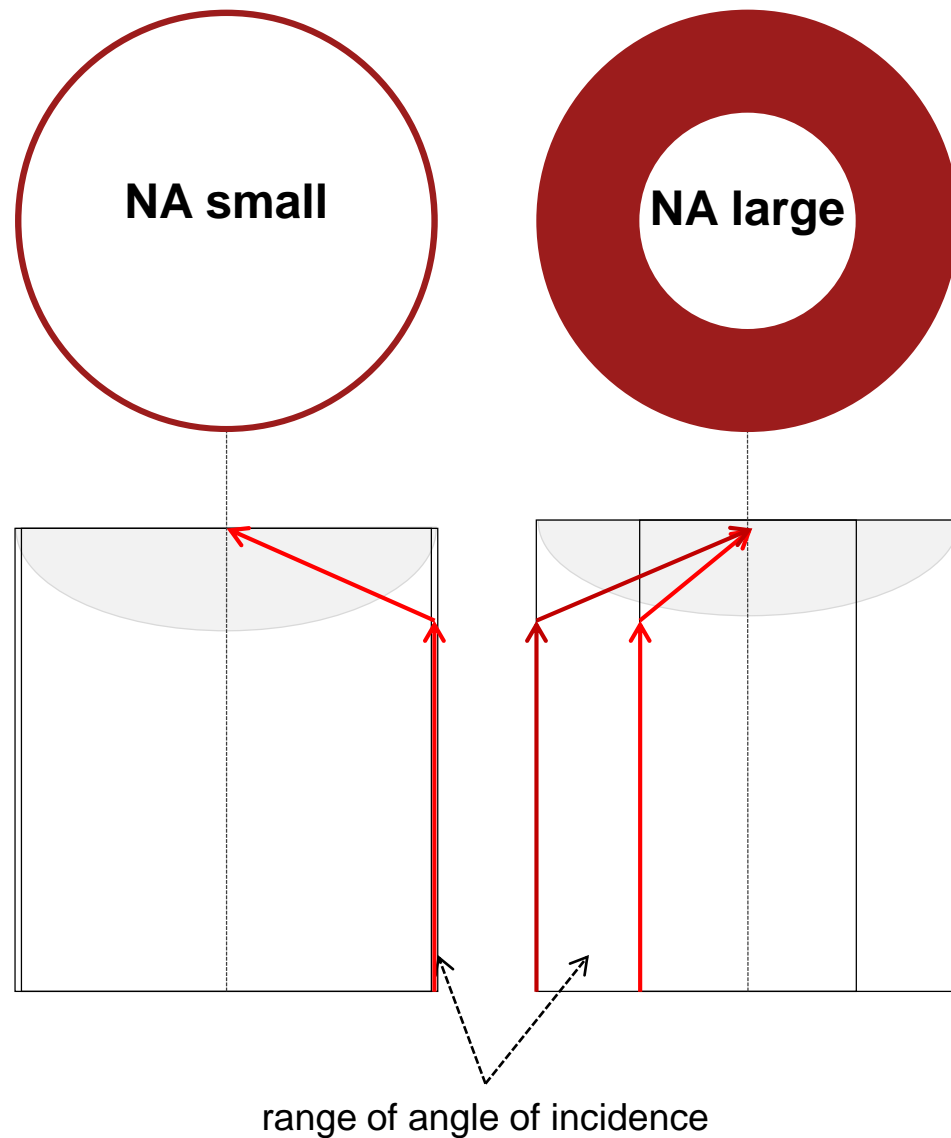
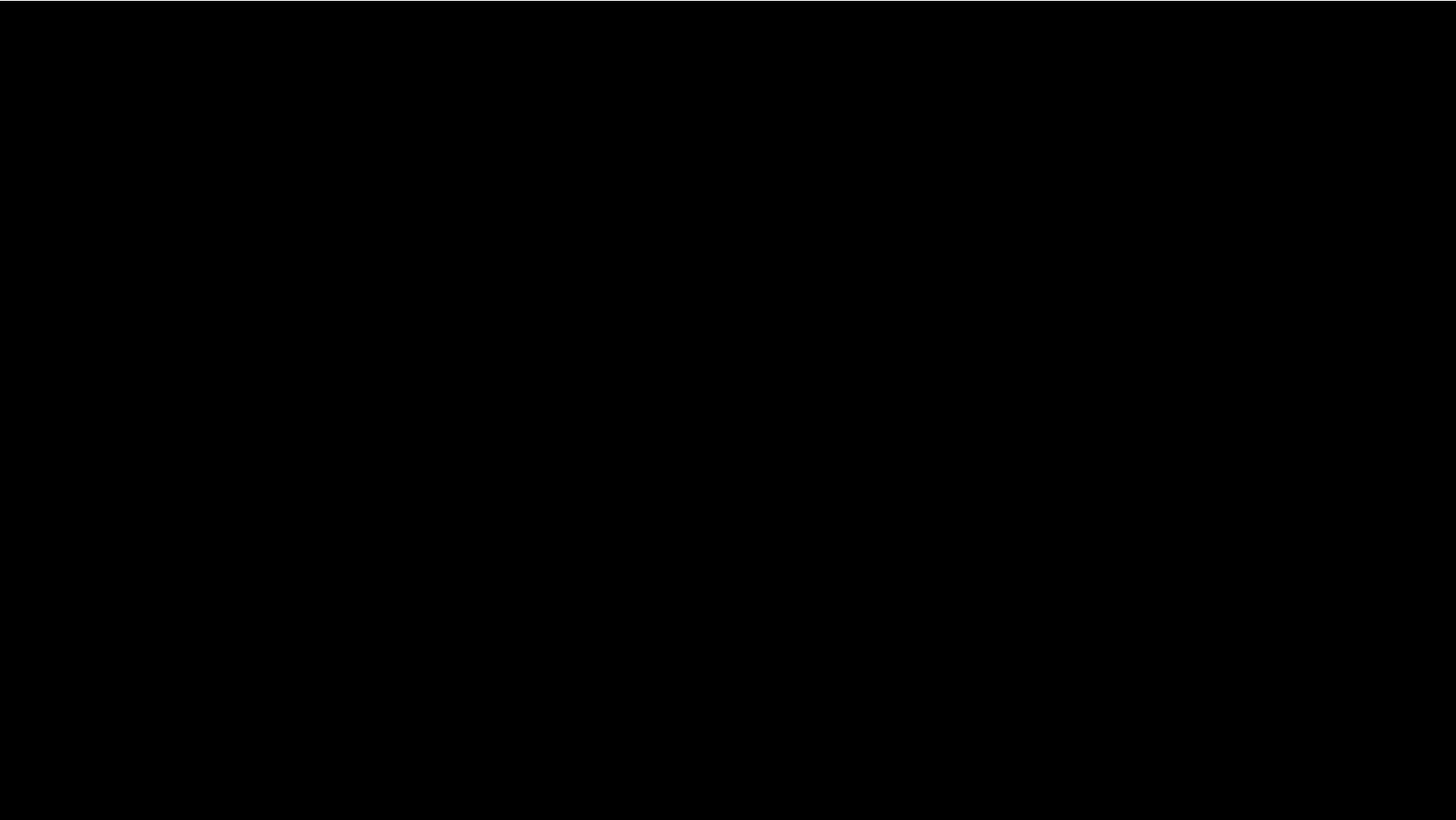**minimum NA**   **available NA**

NA = 1.4
2 %

NA = 1.49
11 %

NA = 1.65
27 %

NA small

NA large

range of angle of incidence

$n_{glass(1)} = 1.515$
$n_{cell(2)} = 1.38$

$\alpha = 79.6°$

$\alpha = 65.6°$

angle of incidence (°)

NA

What TIRFM can/cannot do?

☺ ☹ <u>study structures/processes **near/at a surface**</u>
- sample is either in focus our out-of-focus, it is not a question of where you focus
- TIRFM: single layer – confocal microscopy: any layer

→ ☺ **cylindrical lens, Z (axial) information**

☺ <u>**improved axial resolution $d_z \sim 100\ nm$**</u>
- depending on λ, n, NA

☹ <u>**classic lateral resolution $d_{x,y} \sim 200\ nm$**</u>

→ ☺ **TIRFM + SIM $d_{x,y} \sim 100\ nm$**

→ ☺ **TIRFM + STORM single molecule imaging $d_{x,y} \sim 10\ nm$**

☺ <u>**sample**</u>
- living/fixed cells adherent, recombinant proteins
- surface chemistry!
- coverslip, #1, 1.5!!
- no special need for sample preparation

☺ <u>**less cytotoxicity**</u>
- longer time-lapse movies

☺ <u>**does not require special fluorophore**</u>

☺ <u>**relatively easy to operate**</u>

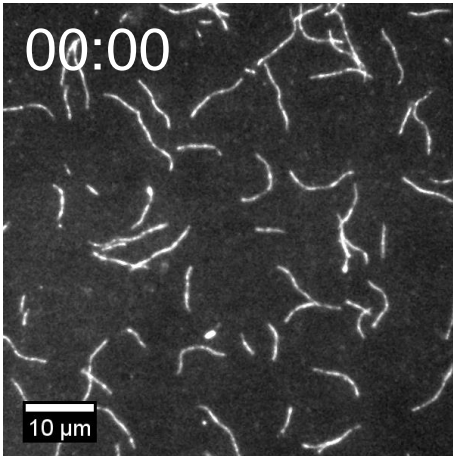☺ <u>can be **combined with other approaches**</u>
- FRAP, FRET, AFM, microfluidics, micropatterning

☹ <u>**irregular illumination field can degrade image quality**</u>
interference fringes, scattering, shadowing by cellular structures, improper alignment

→ ☺ **spinning TIRFM**
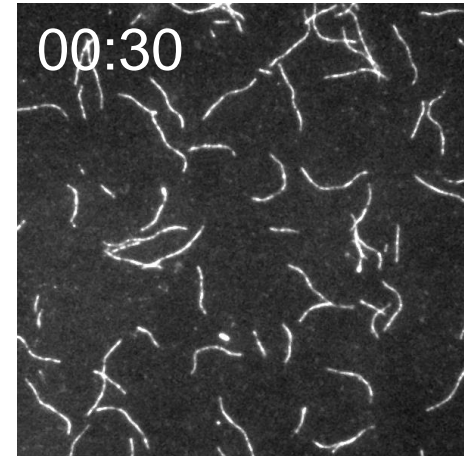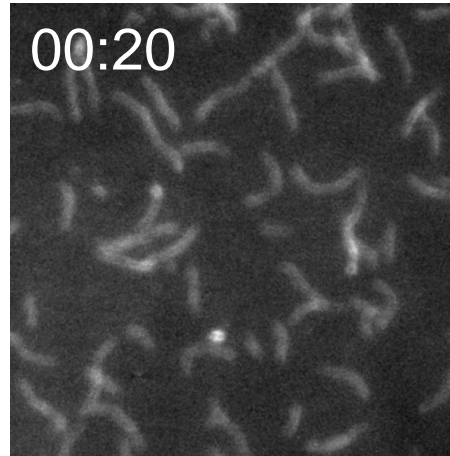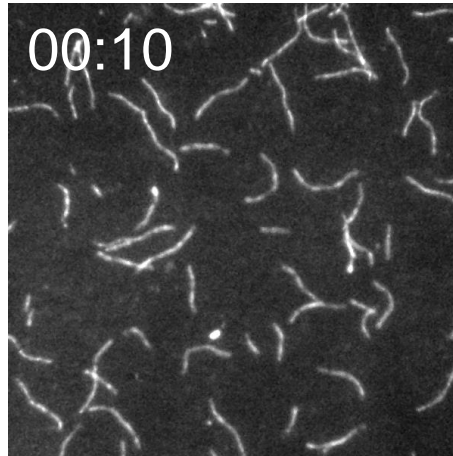
focus drifting

cause:

The thin optical section imaged with TIRF makes it **particularly sensitive to small changes in focus**, which degrade image quality.

solve:

- manually adjust focus
- focus-maintaining solution, Z drift compensator

below supercritical regime

supercritical regime

contamination of image with propagating light

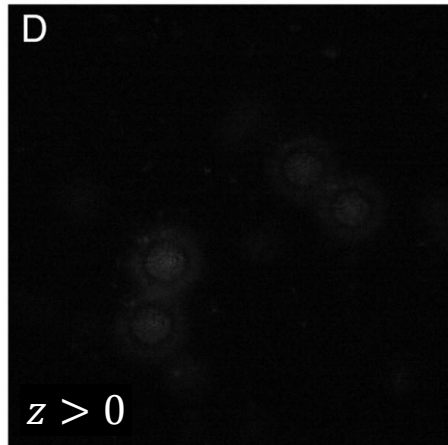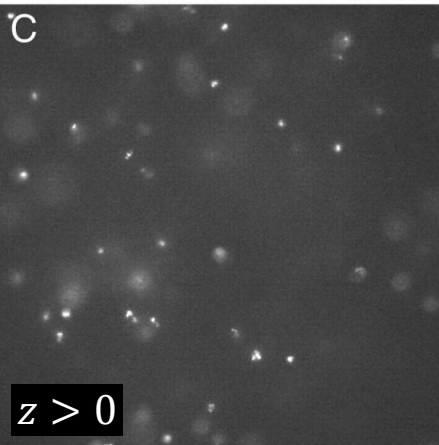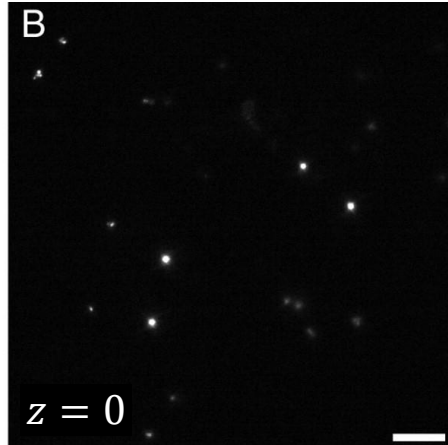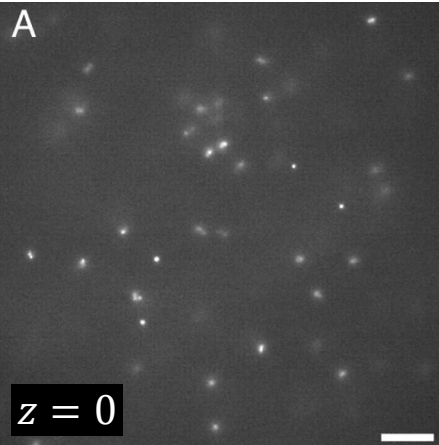if the image can be focused in more than one z plane, then propagating light is a problem

cause:

- improperly aligned laser (subcritical angle of incidence)
- tilted coverslip
- scattering by the sample

solve:

check the hardware; parameters of the TIRFM setup

- vary the incidence angle
- align laser

interference fringes

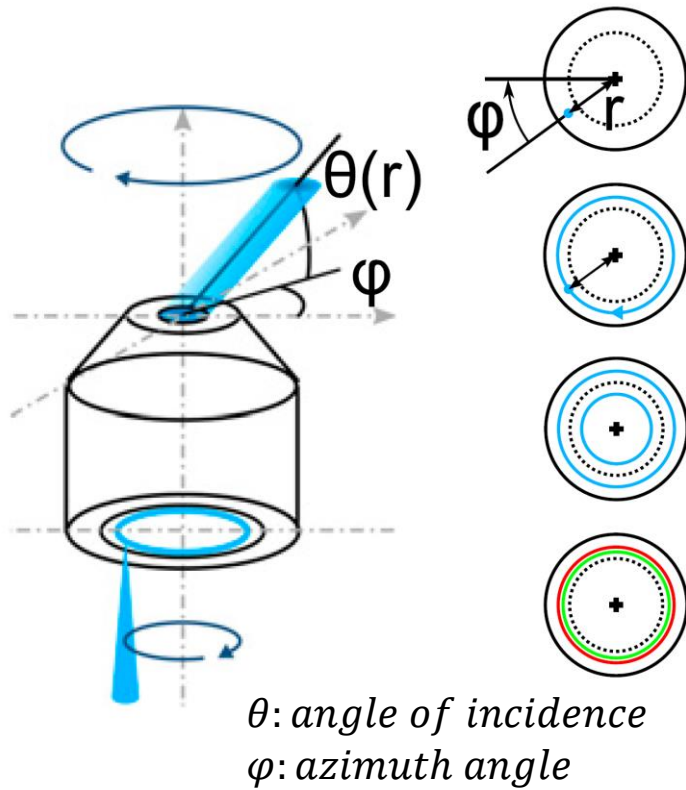alternating light-dark pattern of excitation intensity at the sample plane is apparent

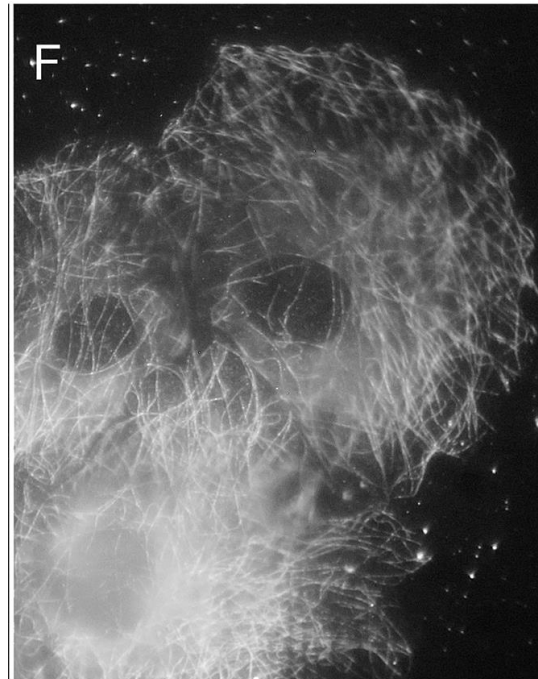*cause*:
- optical imperfections in the beam path

solve:
- cleaning dust off optical surfaces
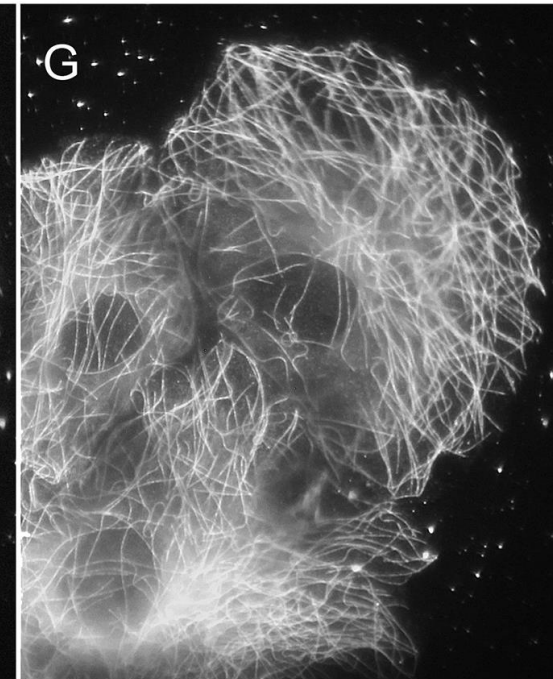- use dedicated optical elements
- spinning TIRFM
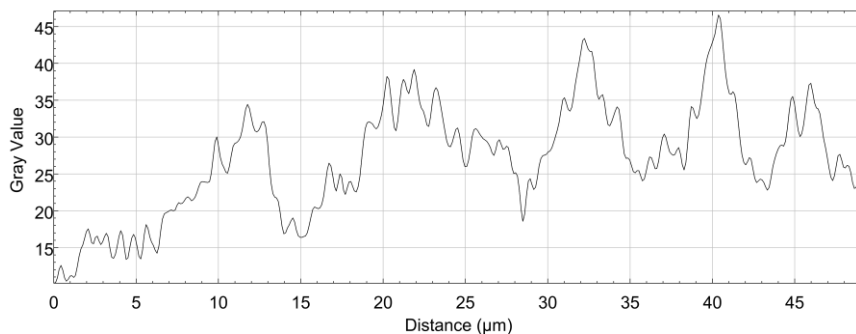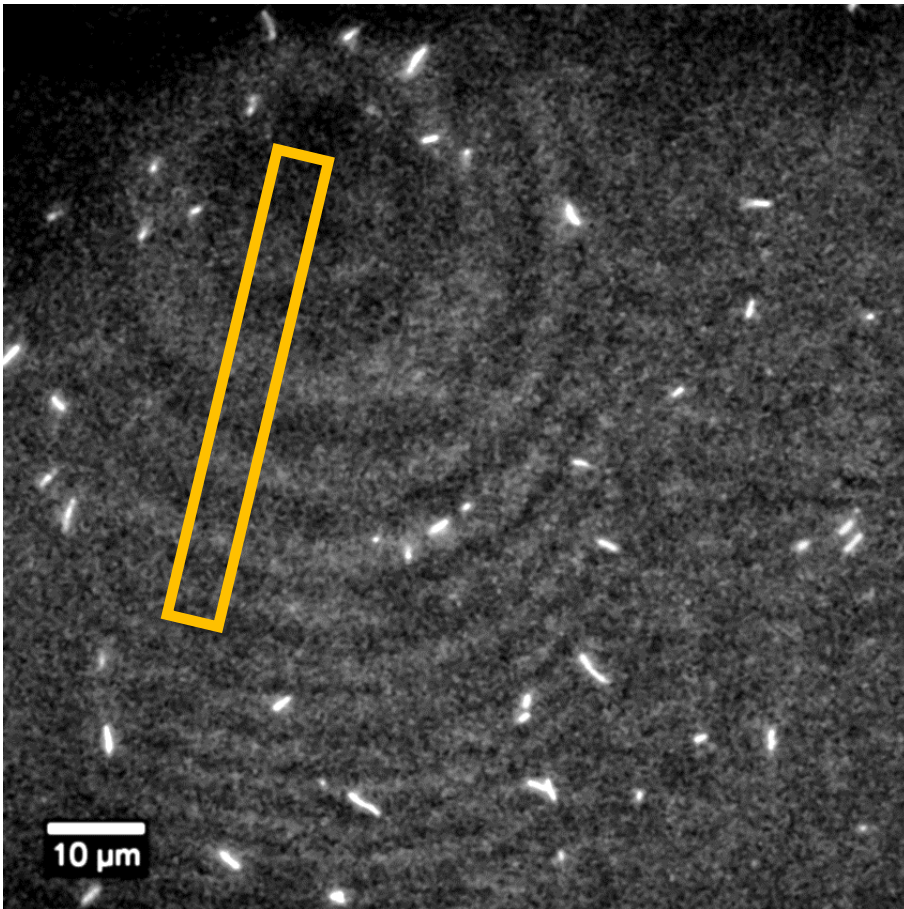
stationary-spot TIRF   spinning-spot TIRF

θ: *angle of incidence*
φ: *azimuth angle*

(F-G) COS-7 cells expressing tubulin (EGFP), stationary-spot TIRF (F), spinning-spot TIRF (G).
Utilizes a pair of galvanometer mirrors to **spin the laser spot around a circle at the outer edge of the objective's back focal plane**. Consequently, a collimated beam with fixed polar angle and spinning azimuthal angle illuminates the specimen. The **different shadowing and interference patterns at each angle thus average out during the single-frame exposure time of the camera to produce a substantially uniform illumination field**.
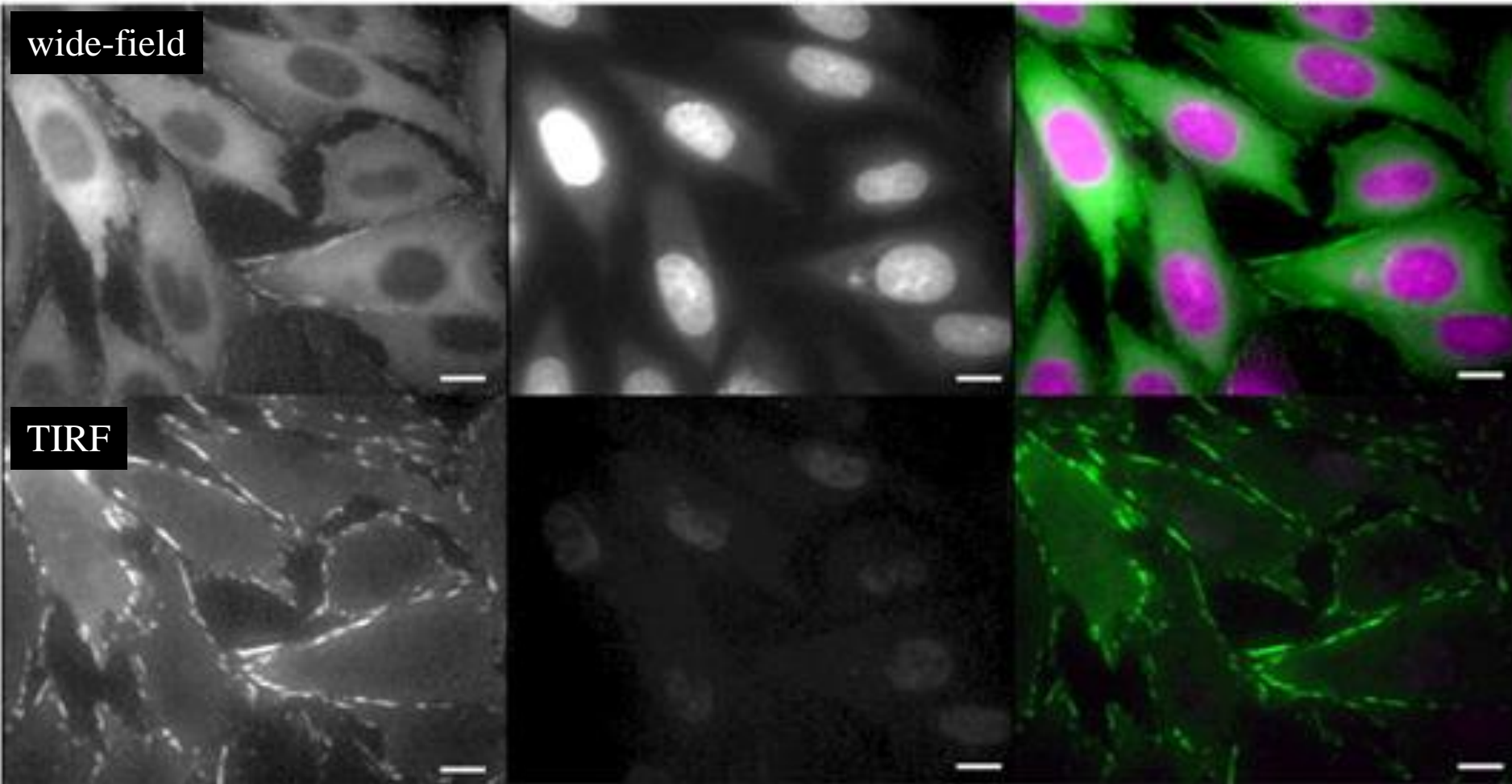**Irregularities in the illumination field averages out.**

Ellefsen, K. L., Dynes, J. L., & Parker, I. (2015). Spinning-Spot Shadowless TIRF Microscopy. PLoS ONE, 10(8), e0136055. http://doi.org/10.1371/journal.pone.0136055

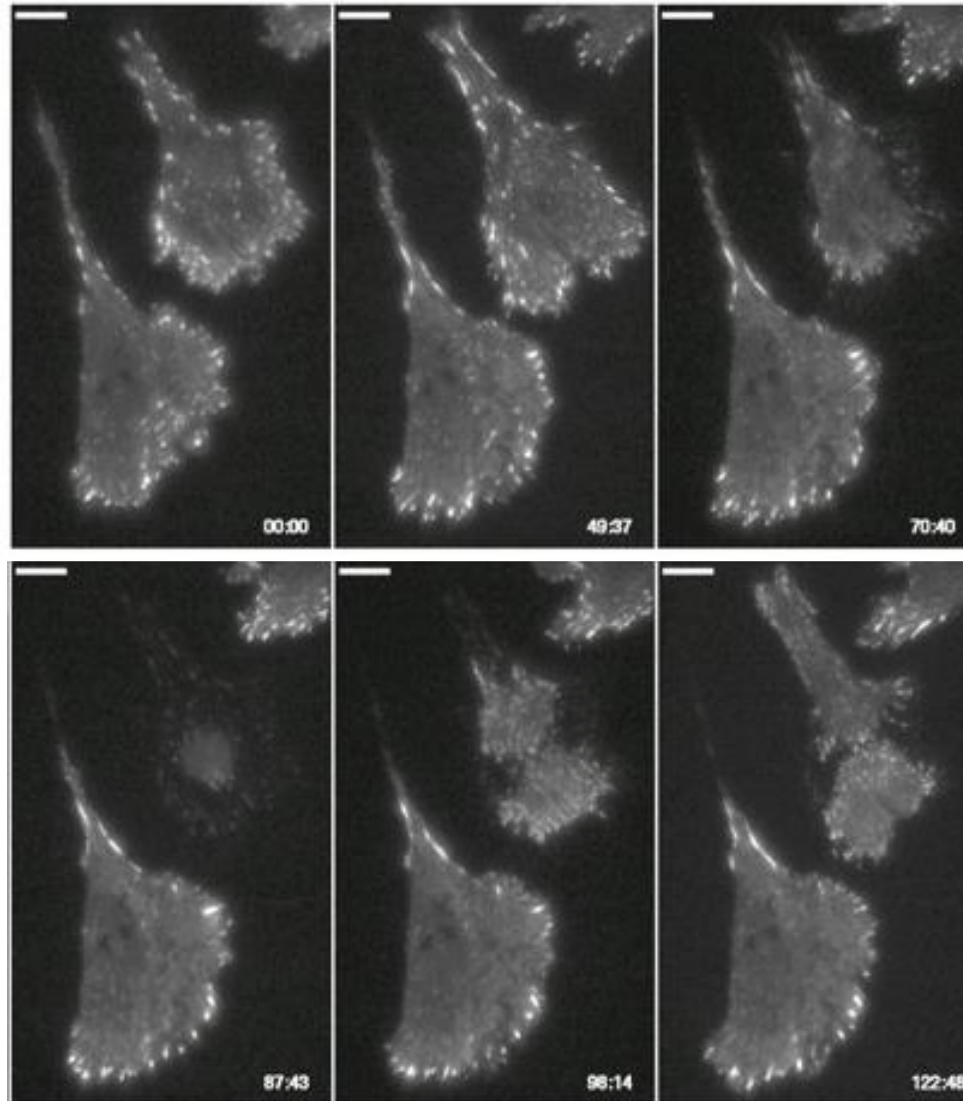# TIRFM – cell biological applications
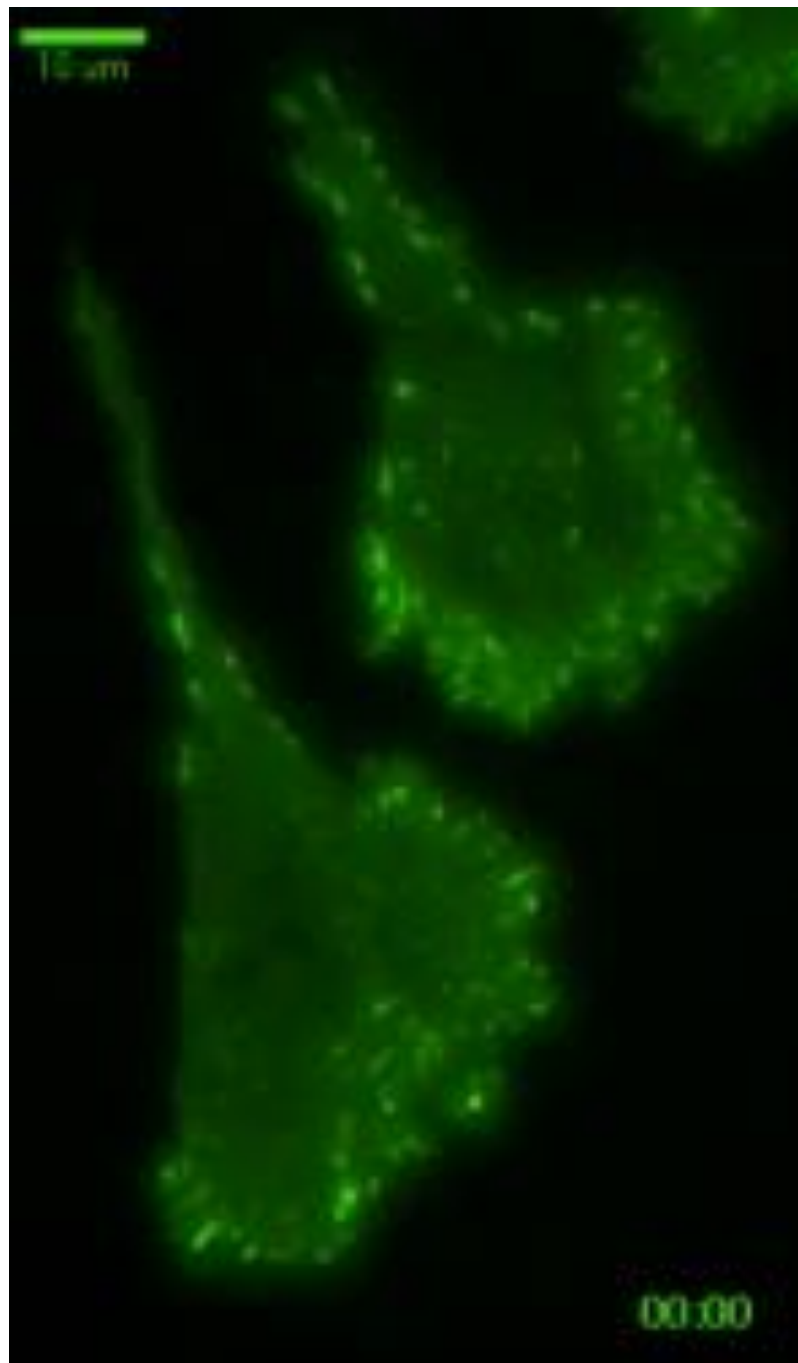

Chinese hamster ovary (CHO) cells, EGFP-paxillin. Scale bars: 10 μm.

PAXILLIN: focal adhesions, cell adherence to extracellular matrix

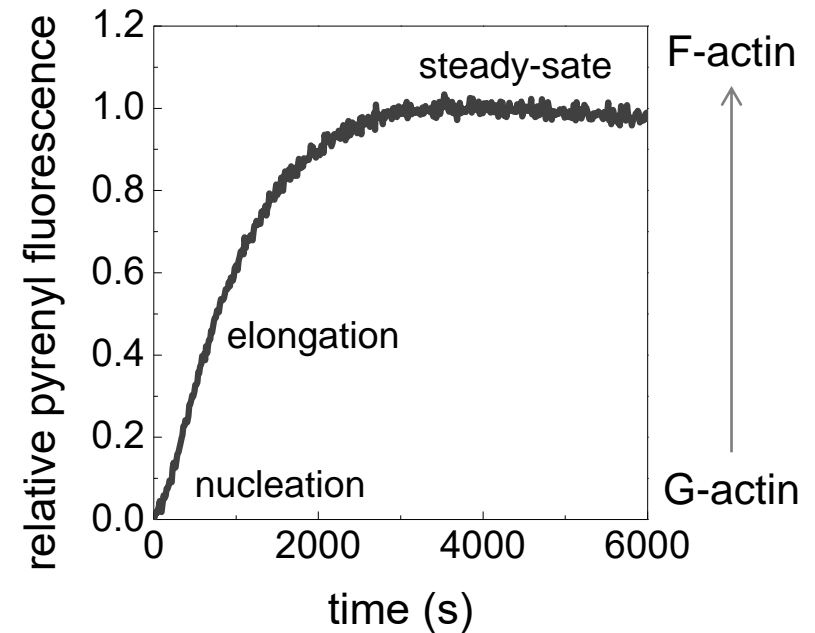**Draq5**: Deep Red Anthraquinone 5, nucleic acid staining

Lack of nuclear/cytoplasmic staining – specificity for the basal cell surface.

TIRF images of Chinese hamster ovary (CHO) cells, EGFP-paxillin. Scale bars: 10 μm.
PAXILLIN: focal adhesions, cell adherence to extracellular matrix
A cell rounds up and disappears from the TIRF field during cell division.

M. M. Frigault, J. Lacoste, J. L. Swift, C. M. Brown *Live-cell microscopy – tips and tools.* Journal of Cell Science 2009 122: 753-767; doi: 10.1242/jcs.033837
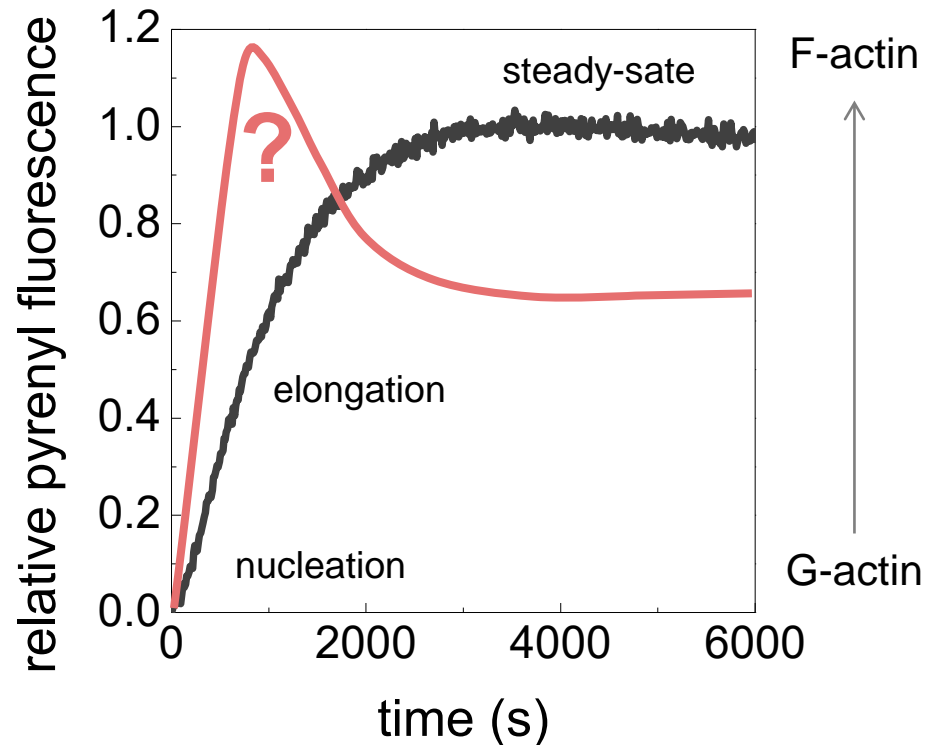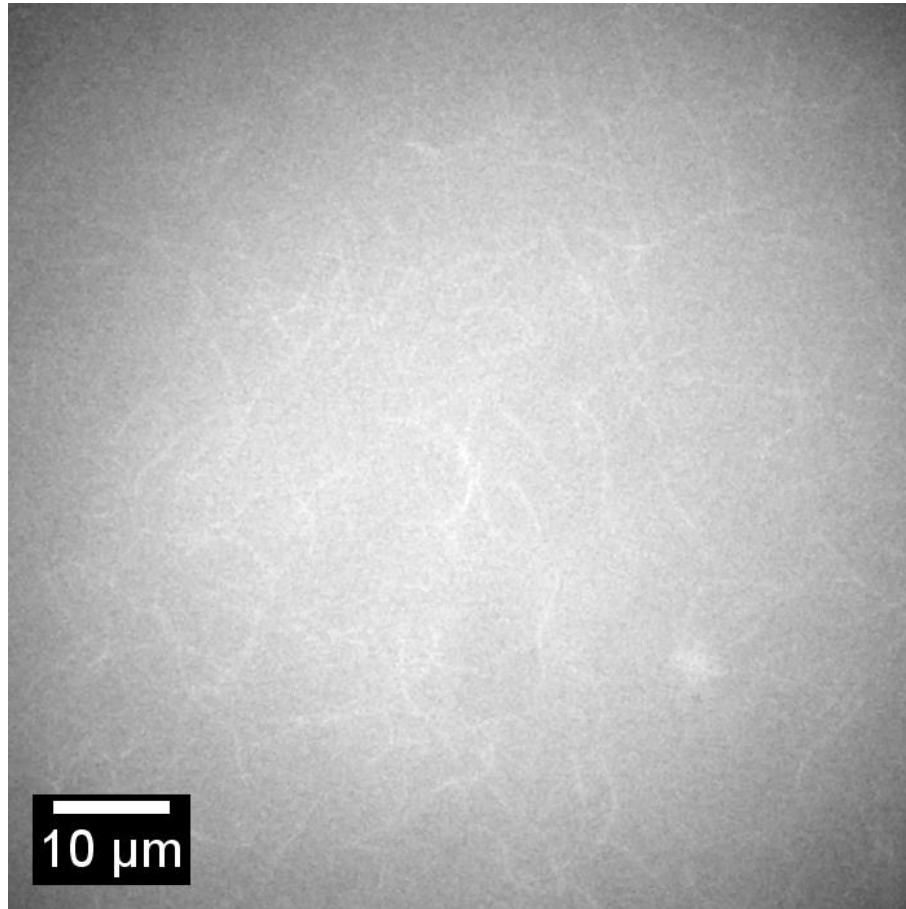
**SPONTANEOUS
ACTIN ASSEMBLY**

$$v = k_+[G_0 - c_c][F] - k_-[F]$$
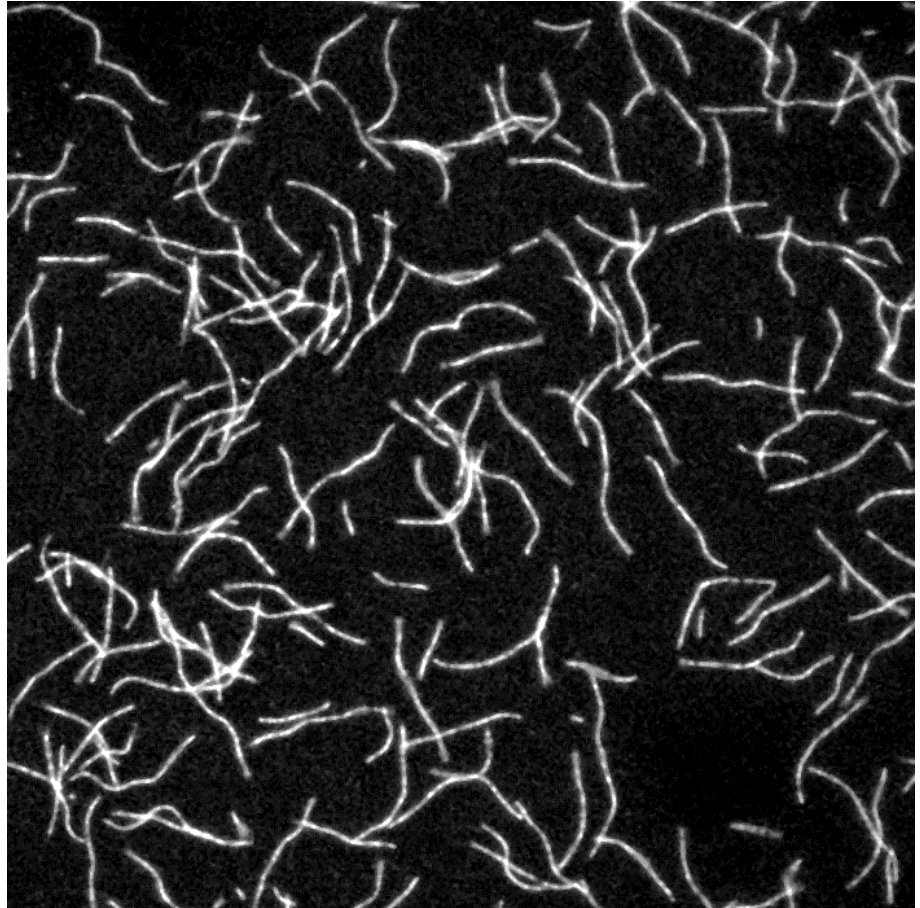
SPONTANEOUS ACTIN ASSEMBLY
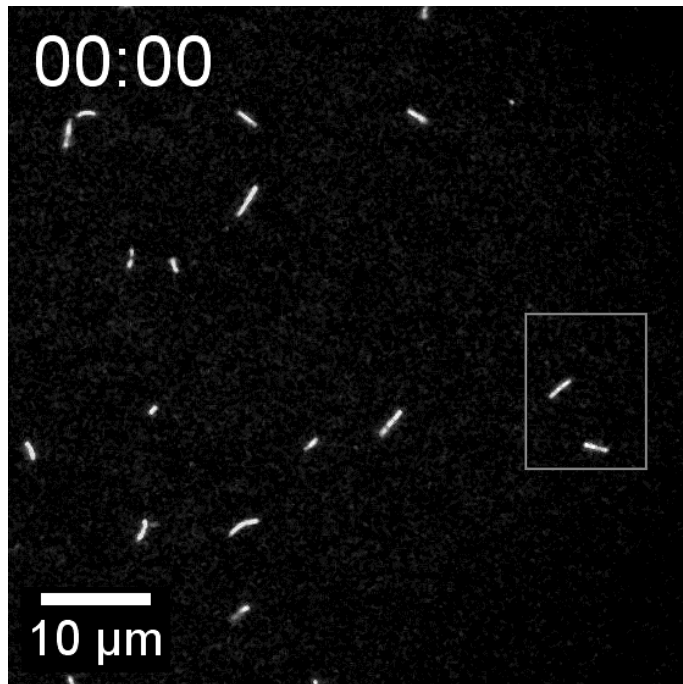
$$v = k_+[G_0 - c_c][F] - k_-[F]$$

Epi

TIRF

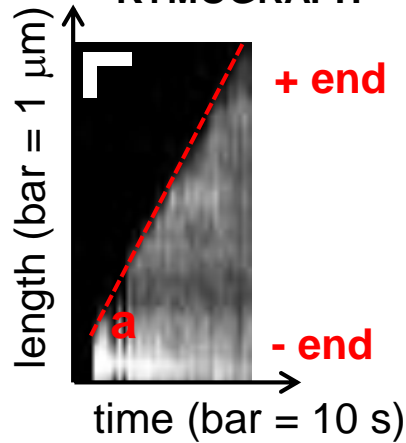Actin polymers - microfilaments in vitro (488 nm)

POLYMER BIOCHEMISTRY – ACTIN, MICROTUBULE DYNAMICS
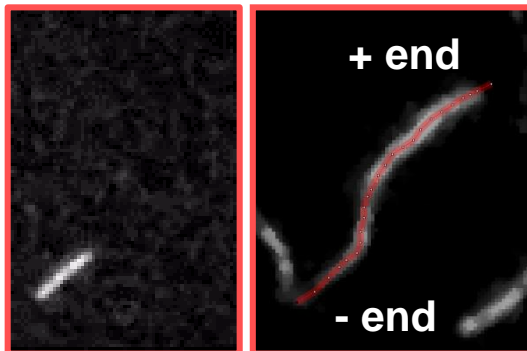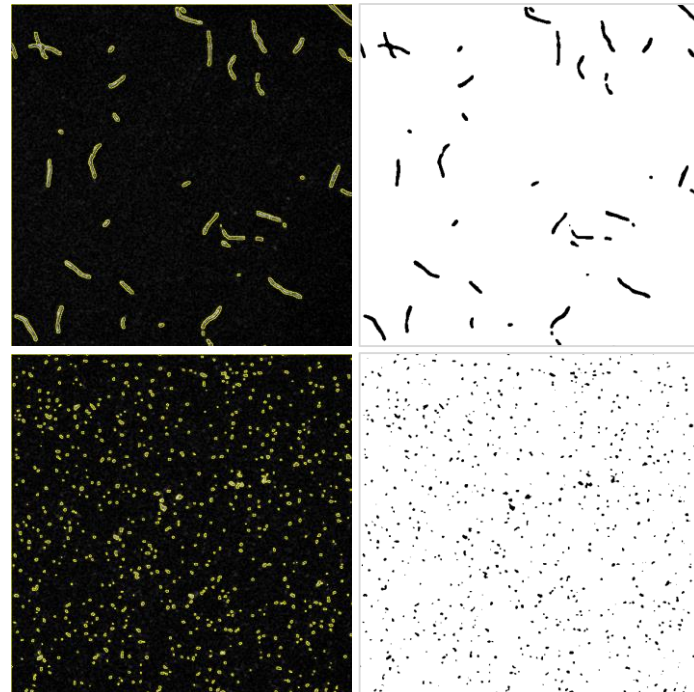
SPONTANEOUS GROWTH OF ACTIN FILAMENTS

10 µm

AlexaNHS488-ACTIN (10 % LABELED)
491 nm 60xNA1.45 time = min : s
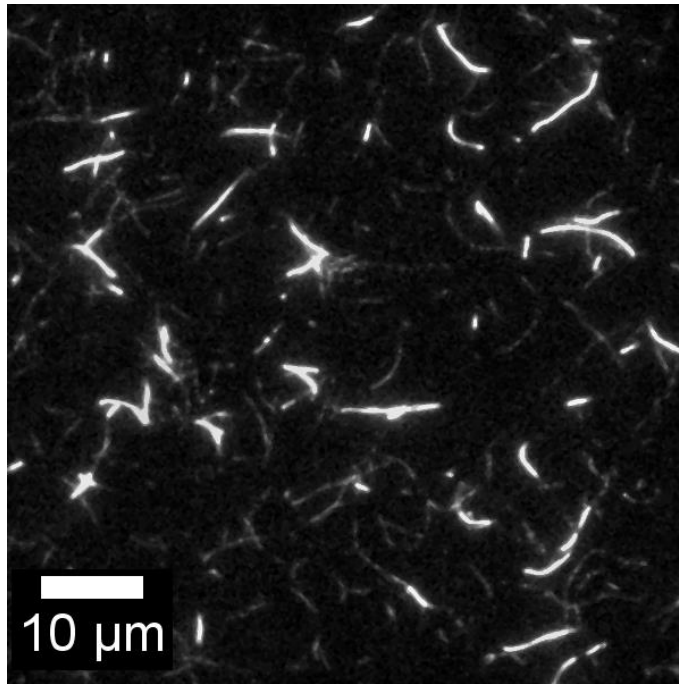t = 100 ms, I = 10% (P = 15 mW) HamamatsuCCD

KYMOGRAPH

length (bar = 1 µm)

+ end

- end

a

time (bar = 10 s)

$$v_{elongation} = tan\alpha = \frac{\Delta length(\mu m)}{\Delta time\ (s)} = \frac{\Delta length * 370(su)}{\Delta time\ (s)}$$

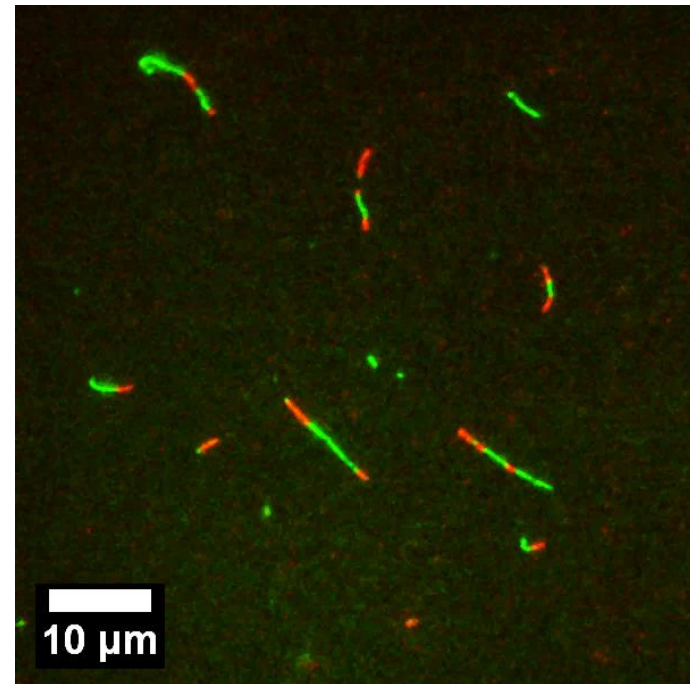$$v_{elongation} = k_+[G - c_c] \rightarrow k_+ = 11\ \mu M^{-1}s^{-1}$$

SPONTANEOUS ASSOCIATION OF INDIVIDUAL POLYMERS INTO HIGHER ORDER STRUCTURES
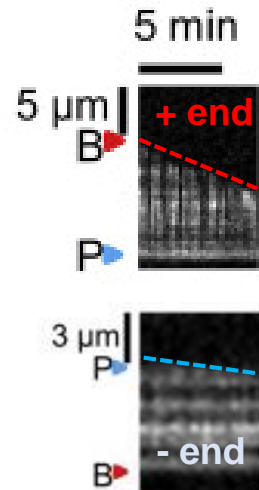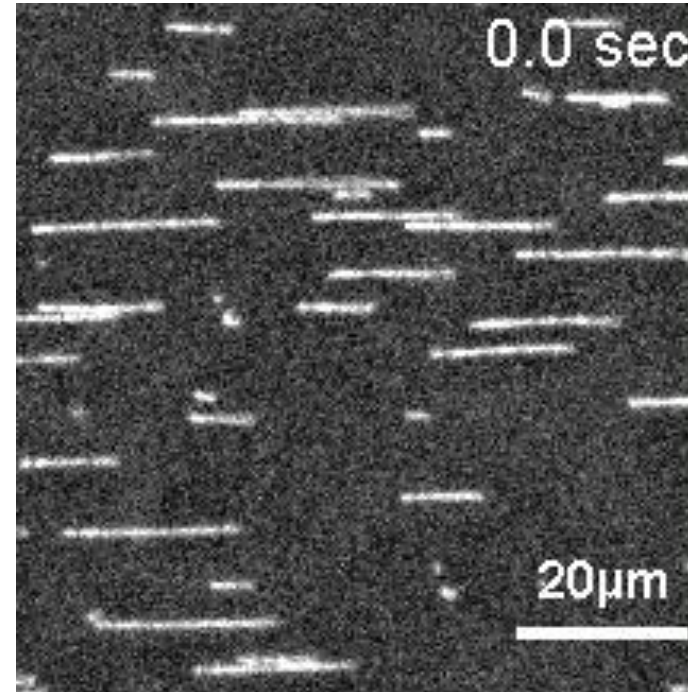
SIDEWISE ASSOCIATION
cross-linking/bundling

ENDWISE ASSOCIATION
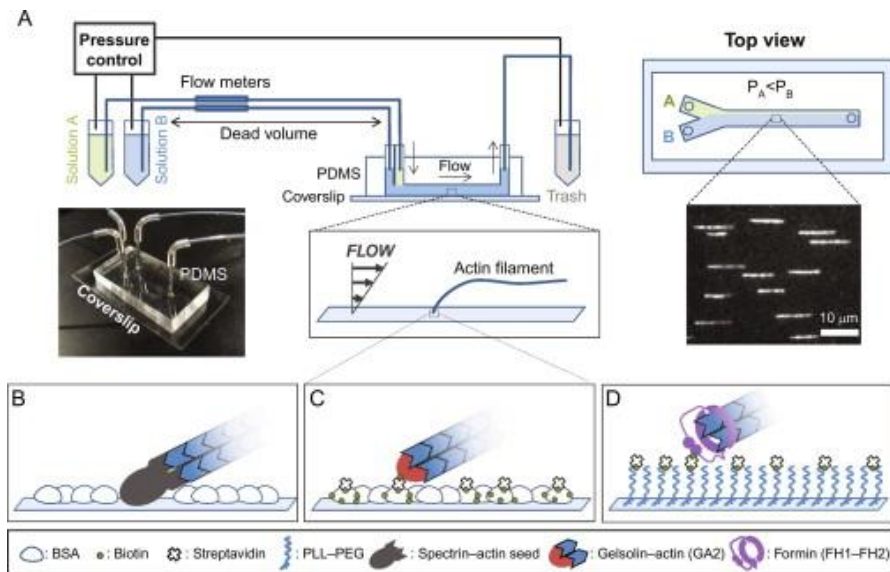**annealing**

radial thickening

lateral growth

Bugyi B. Muscle Contraction a Hungarian Perspective 2018

SPONTANEOUS DEPOLYMERIZATION
DISSOCIATION OF INDIVIDUAL SUBUNITS FROM POLYMER ENDS

MICROFLUIDICS-ASSISTED TIRFM
OBSERVATION OF THE DISASSEMBLY OF
INDIVIDUAL ACTIN POLYMERS

$$k_{OFF}(barbed\ end, ATP) = 1.4\ s^{-1}$$
$$k_{OFF}(pointed\ end, ADP) = 0.25\ s^{-1}$$

Shekar S. et al. Current Biology 2017, Carlier MF. et al. Methods in Enzymology 2012

TIRFM – cell biological applications

Glucose uptake
10 % adipose tissue, 90 % muscle
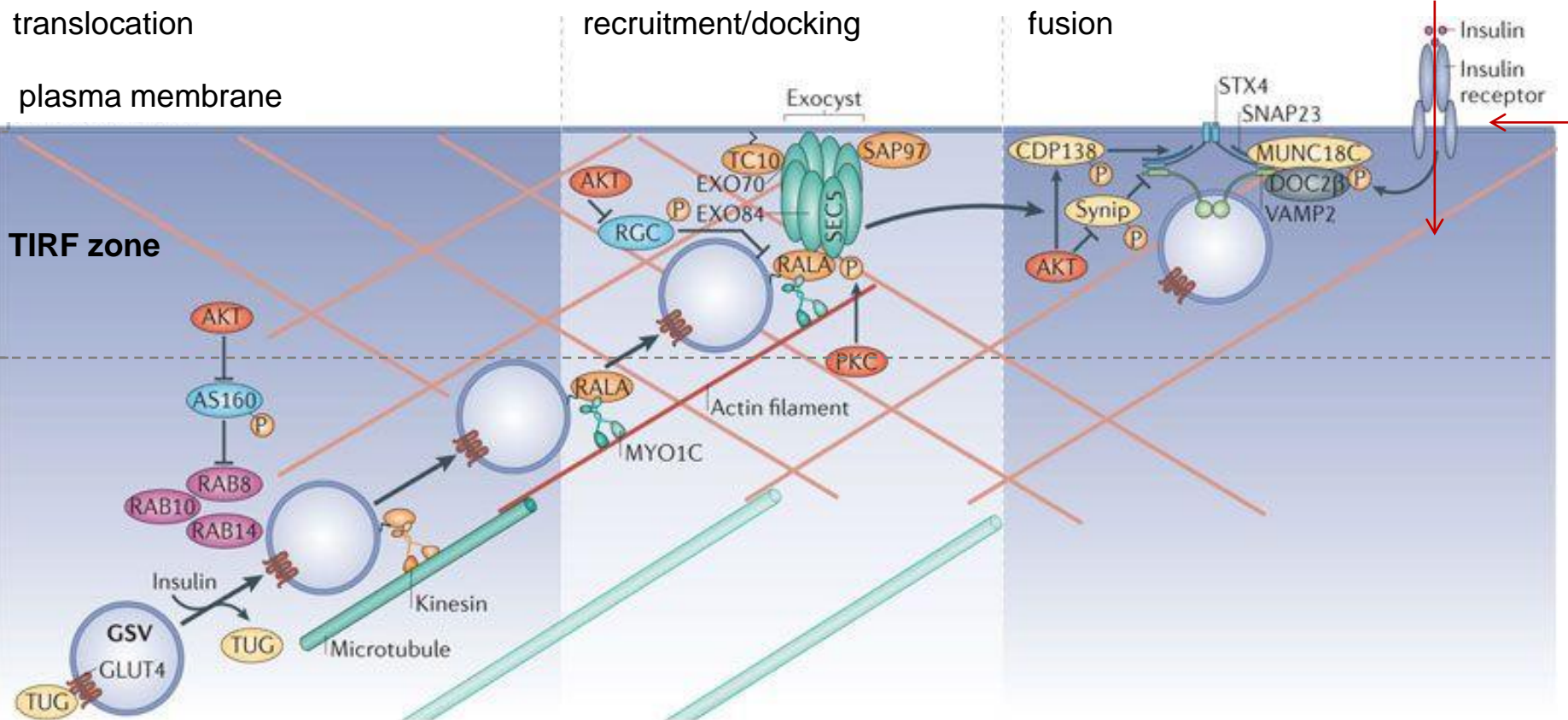insulin resistance, type2 diabetes

insulin

glucose

translocation

recruitment/docking

fusion

plasma membrane

TIRF zone

glucose trasporter (GLUT4)
intracellular storage sites

TIRFM: GLUT4 vesicles near or at the plasma membrane
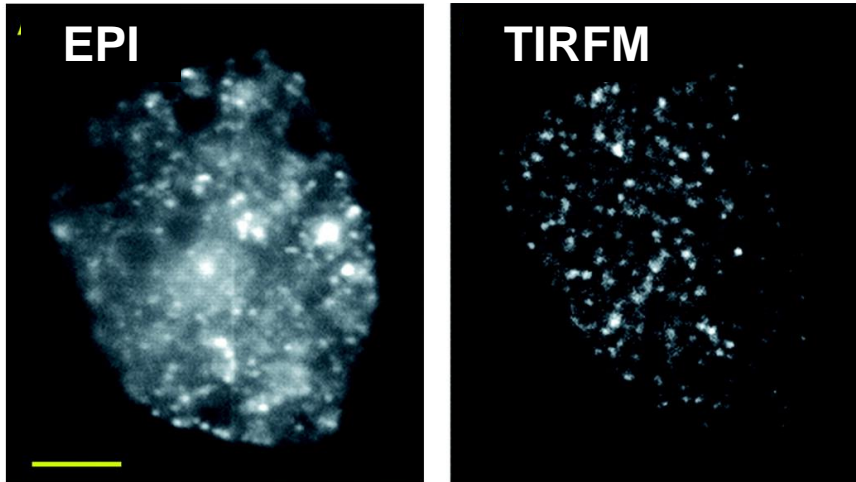
3T3-L1 adipocytes, GFP-GLUT4

Huang et al. Insulin Stimulates Membrane Fusion and GLUT4 Accumulation in Clathrin Coats on Adipocyte Plasma Membranes. Molecular Biology of the Cell 2011
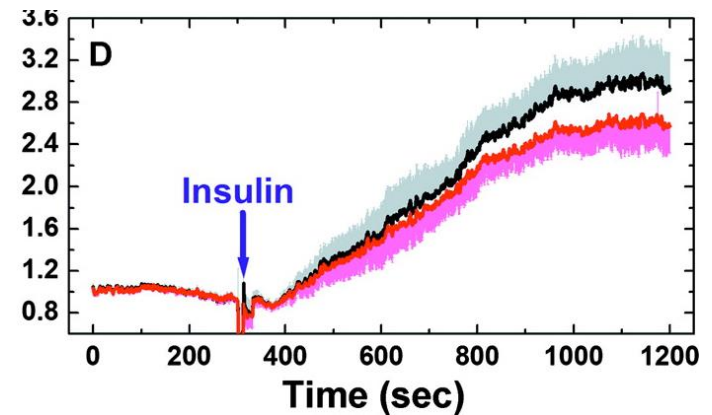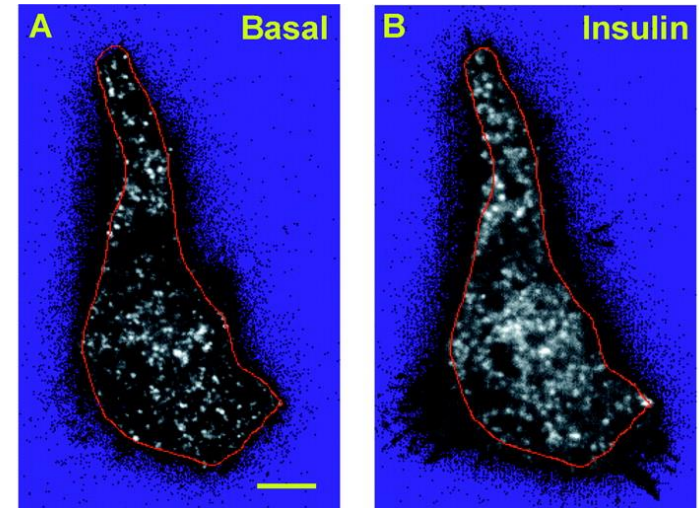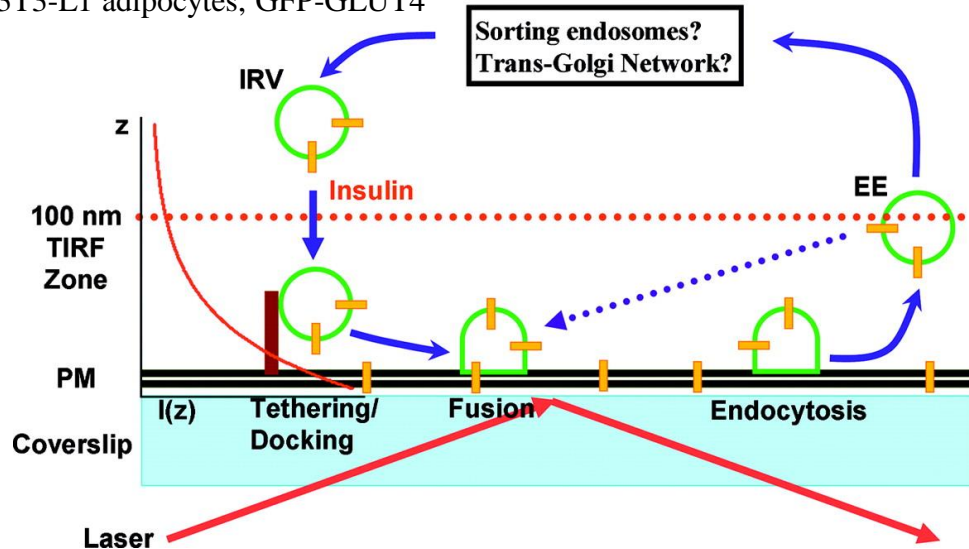
TIRFM: GLUT4 vesicles near or at the plasma membrane
**EPI-IF: inserted into the plasma membrane**
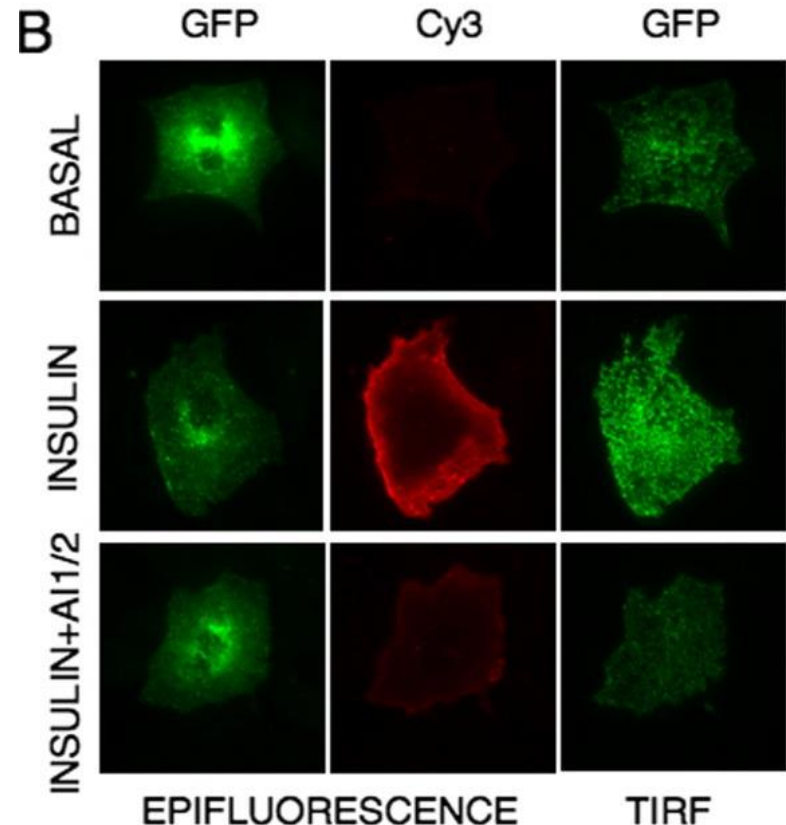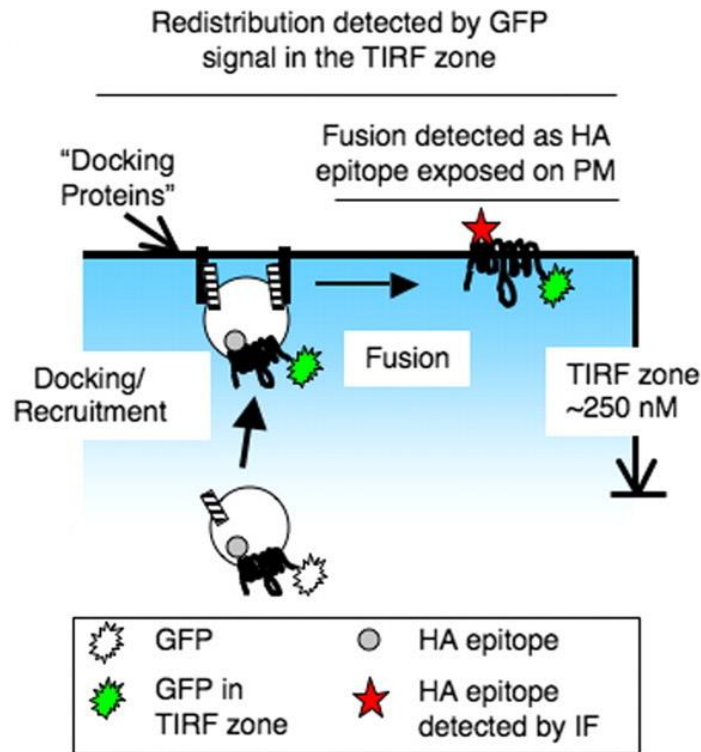
HA-GLUT4-GFP fluorescence in basal or insulin-stimulated adipocytes
The TIRF assay measures redistribution to within 250 nm of the PM independently of whether the GLUT4 is inserted into the PM (that is, independently of vesicle fusion). **GFP = in the vicinity and inserted into the plasma membrane**
In the epifluorescence mode, translocation is measured as the exposure of the HA-epitope of HAGLUT4-GFP on the surface of intact adipocytes, which is measured by IF. **CY3 = inserted into the plasma membrane**

Gonzalez E. et al. Insulin Signaling Diverges into Akt-dependent and -independent Signals to Regulate the Recruitment/Docking and the Fusion of GLUT4 Vesicles to the Plasma Membrane. Molecular Biology of the Cell 17, 4484–4493, 2006

TIRFM: VAMP2-pHluorin: pHluorin is a pH-sensitive fluorescent protein that becomes much more fluorescent when transferred from an acidic (enclosed in the vesicle lumen) to a neutral (vesicle opens) environment
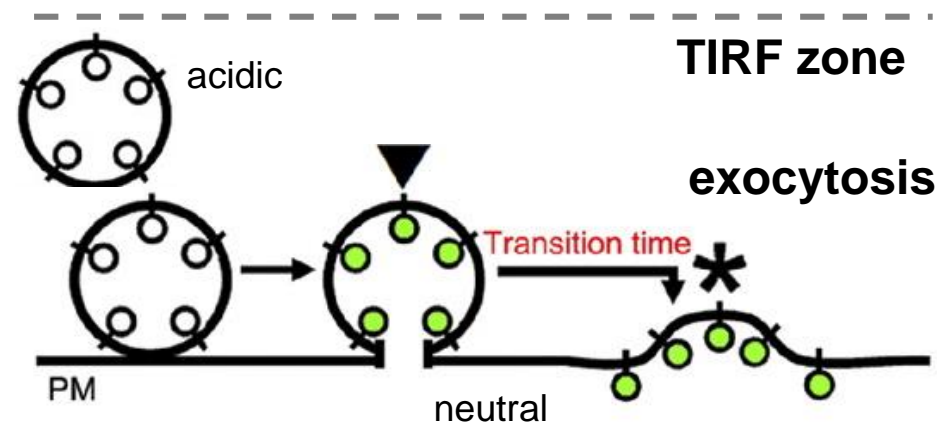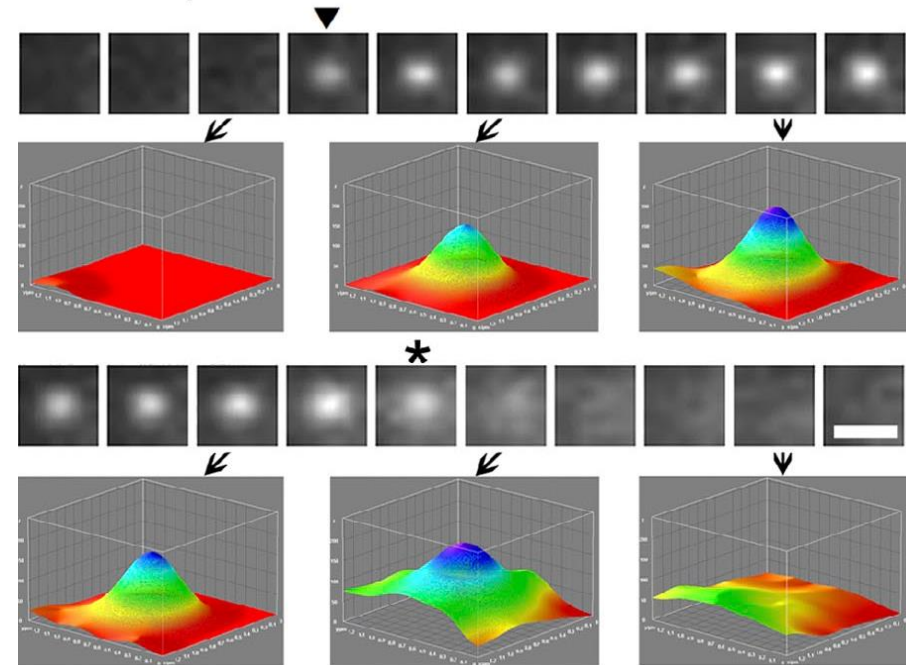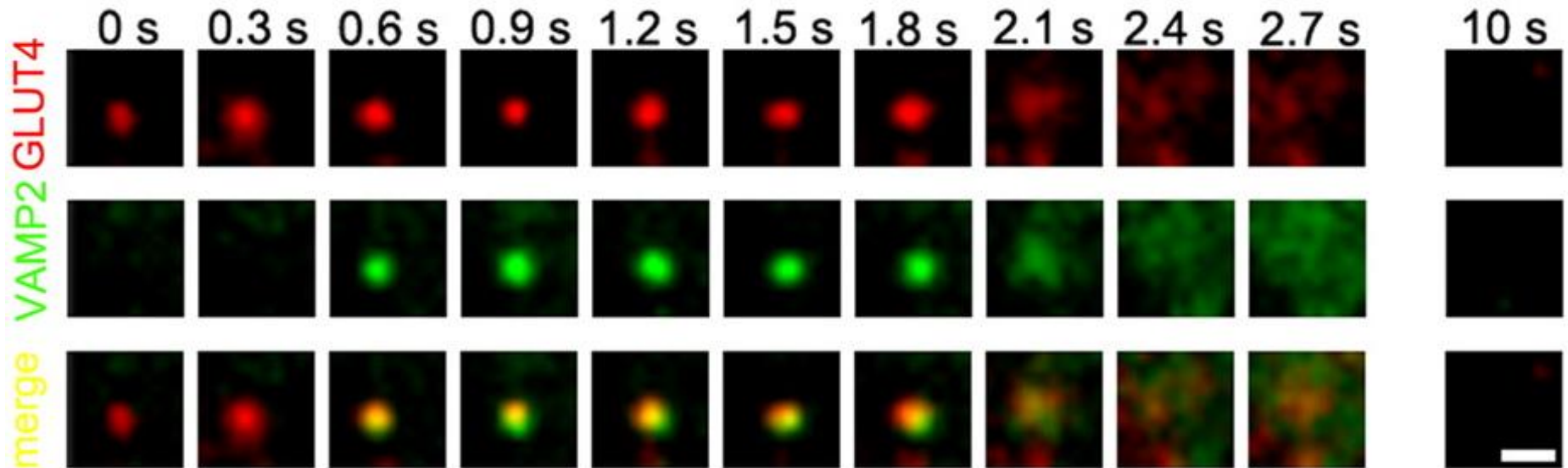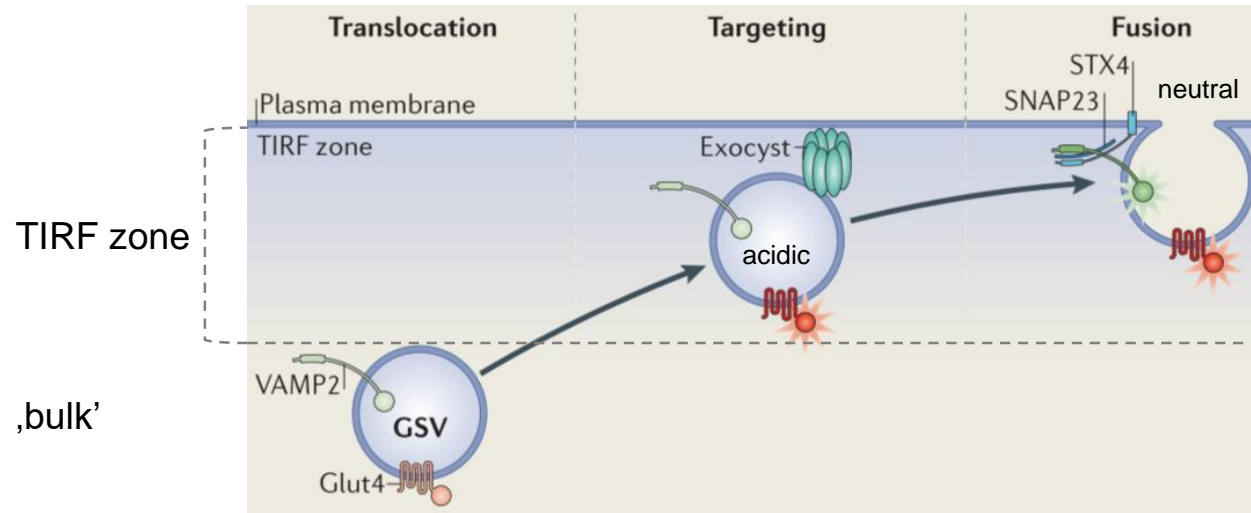

VAMP2-pHluorin fluorescence appears, brightens upon fusion pore formation, and then spreads laterally as the vesicle collapses into the PM

3T3-L1 adipocytes, VAMP2-pHluorin: pHluorin is a pH-sensitive fluorescent protein that becomes much more fluorescent when transferred from an acidic to a neutral environment; GFP-GLUT4

Xu, Y. *et al.* Dual-mode of insulin action controls GLUT4 vesicle exocytosis. *J. Cell Biol.* **193**, 643–653 (2011)
Leto D. et al. Regulation of glucose transport by insulin: traffic control of GLUT4. Nature Reviews Molecular Cell Biology 13, 383–396 (2012)

TIRFM – EVANESCENT WAVE ILLUMINATION

TIRFM, AS A NEAR-FIELD IMAGING METHOD PROVIDES A SURFACE-SELECTIVE ILLUMINATION AND RESOLUTION IMPROVEMENT DUE TO THE UNIQUE PROPERTIES OF THE EVANESCENT FIELD.

EVANESCENT WAVE
- decays exponentially in intensity with increasing distance normal to the surface
- decay length (d) is in the order of the wavelength of the incident light (λ)
- selectively excites fluorophores very near to a solid surface (~ 100 nm)
- eliminates background fluorescence from out-of-focus planes, improves SNR
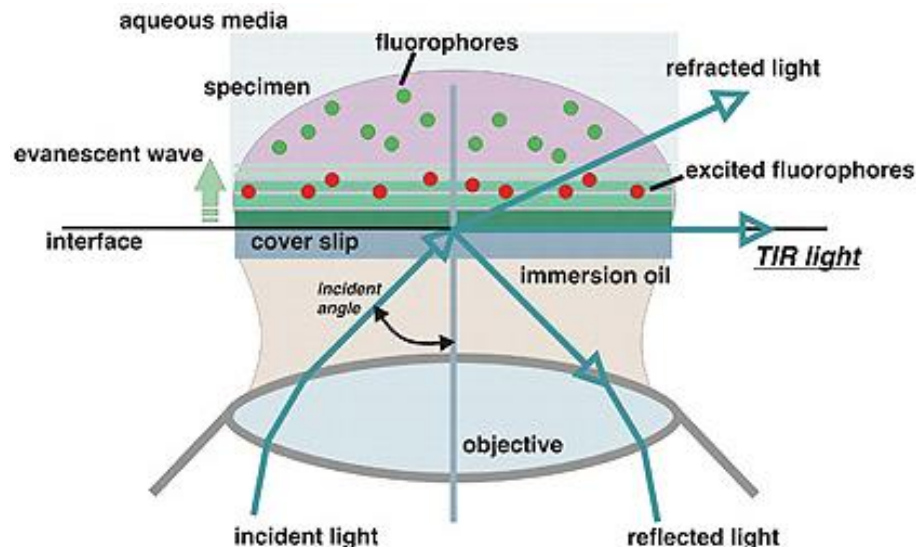
CYTOSKELETAL DYNAMICS GROUP

University of Pécs - Medical School
Department of Biophysics

THE TEAM | PUBLICATIONS | RESEARCH | FUNDING | for STUDENTS | CONTACT

THANK YOU FOR YOUR ATTENTION!

http://cytoskeletaldynamics.wix.com/mysite
http://biofizika.aok.pte.hu

Post-docs
Tamás Huber
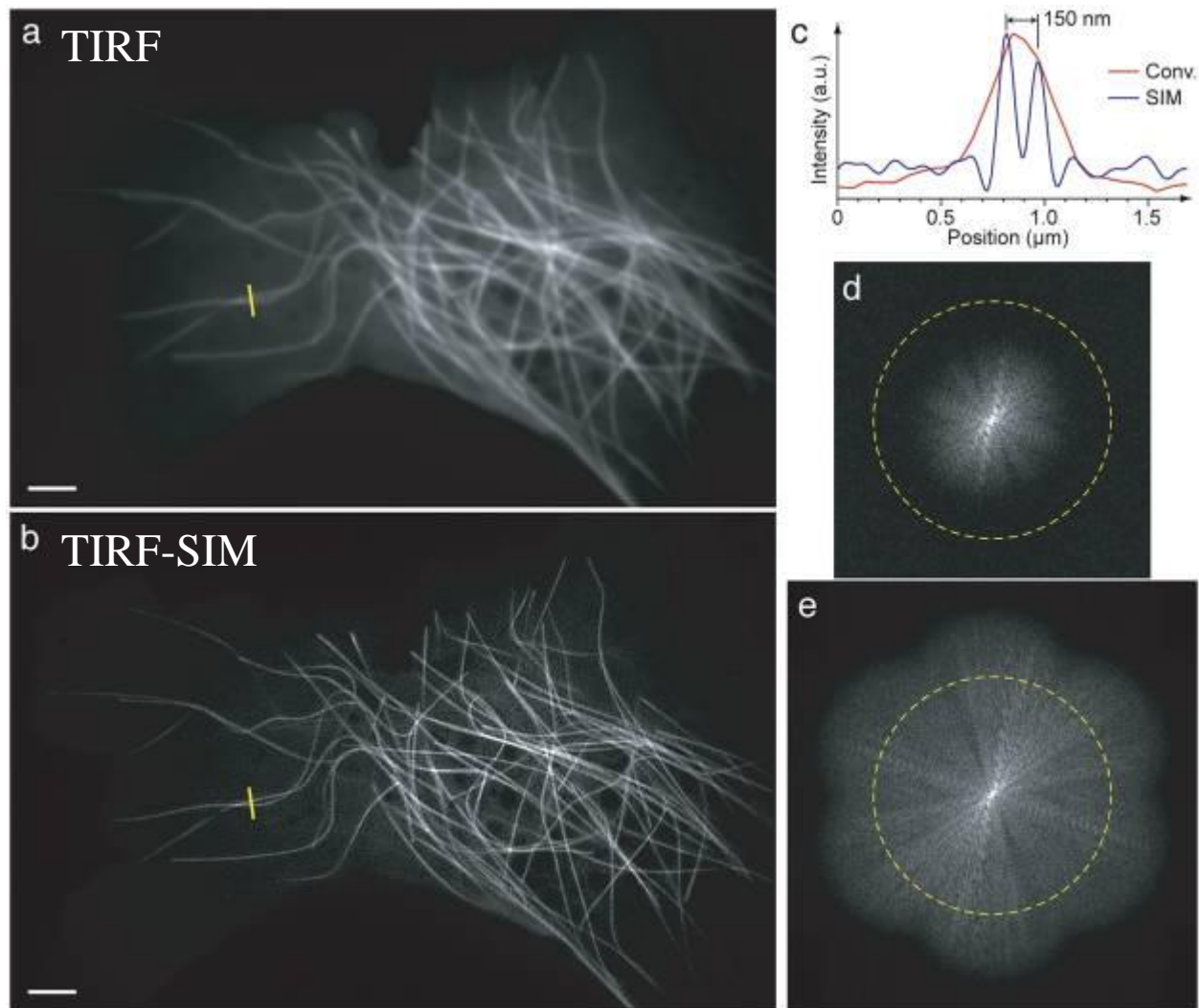Andrea Vig
Péter Bukovics

PhD Students
Réka Pintér
Mónika Tóth
Veronika Kollár
Rauan Shakenov

Péter Gaszler

Comparison of conventional TIRF (**a**) and TIRF-SIM (**b**) images of the microtubule cytoskeleton in a single S2 cell. Scale bar 2 μm. (**c**) Normalized intensity profiles along the yellow lines in (**a**) (red curve) and (**b**) (blue curve). Two microtubules separated by ~150 nm are well resolved in the SIM reconstruction, but not by conventional microscopy. (**d–e**) Fourier transforms of the images in (**a**) and (**b**) respectively. The classical diffraction limit of the objective lens is indicated by a dashed circle of radius 5.96 μm−1.

P. Kner, B.B. Chhun, E.R. Griffis, L. Winoto, M.G.L. Gustafsson *Super-resolution video microscopy of live cells by structured illumination. Nature Methods* vol.6, p.339–342 (2009) Brief Communication
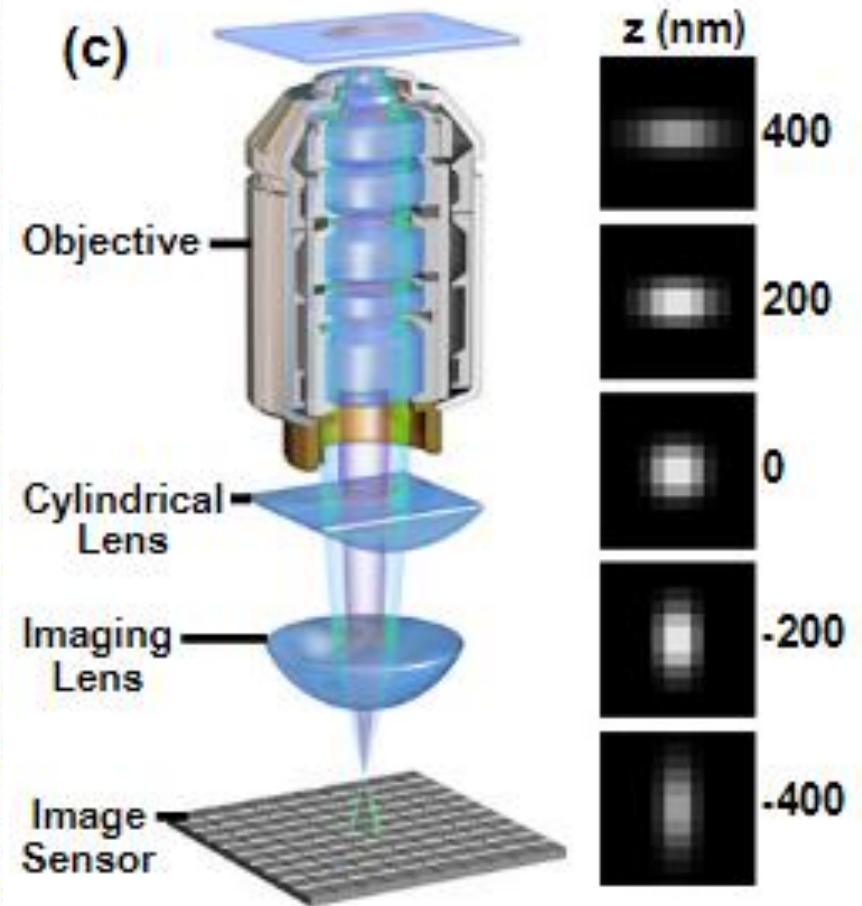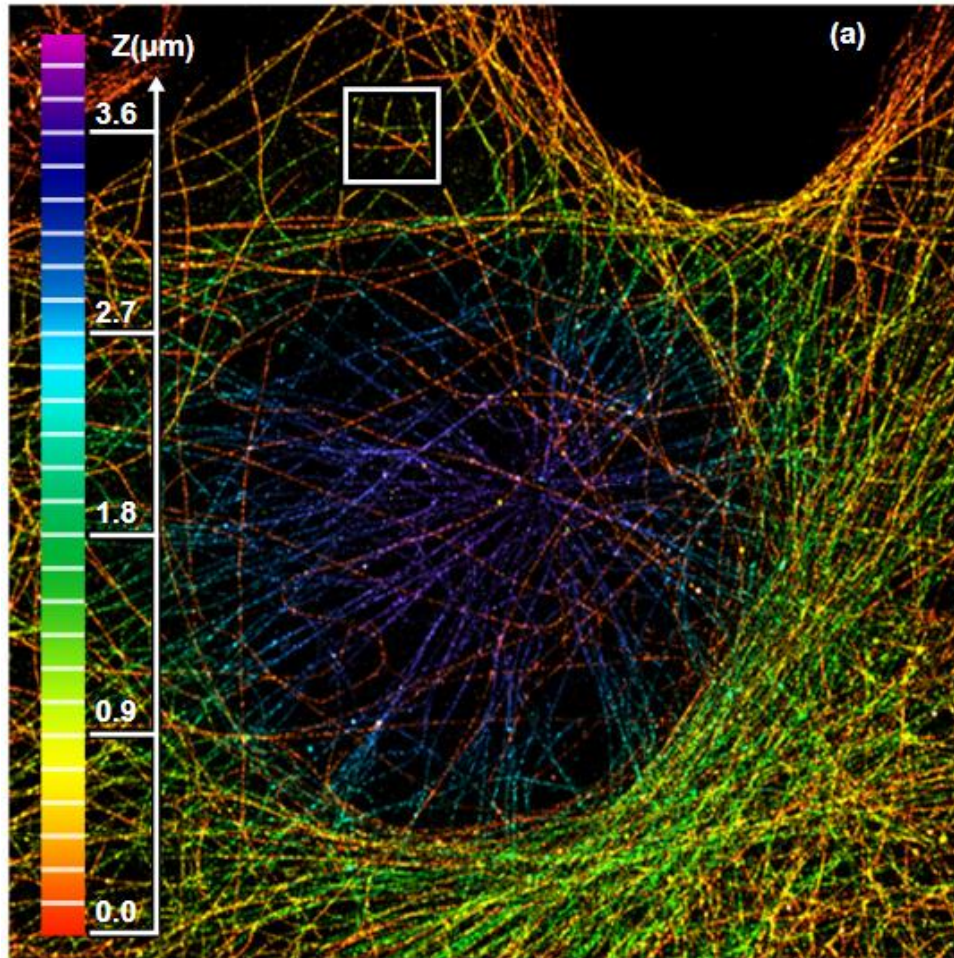
3D STORM image of a BSC-1 cell stained for alpha-tubulin with Alexa Fluor 647 (Nikon N-STORM 4.0). The bar on the left provides the **z position in micrometers (μm) corresponding to each color in the image**. (c) Graphic illustrating the 3D STORM principle. A **cylindrical lens introduces astigmatism, causing the image of single emitters to be stretched in the x or y directions as a function of z position.** Correlation of the magnitude and direction of stretching with a calibration file allows determination of the z position with high precision.
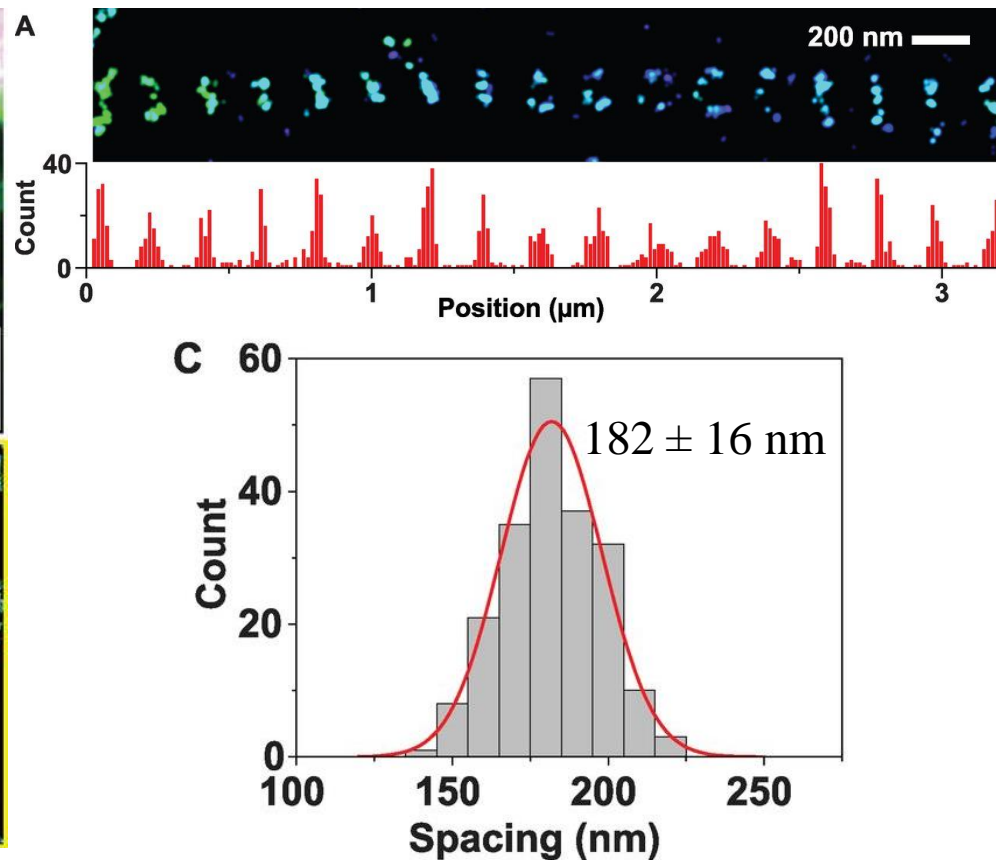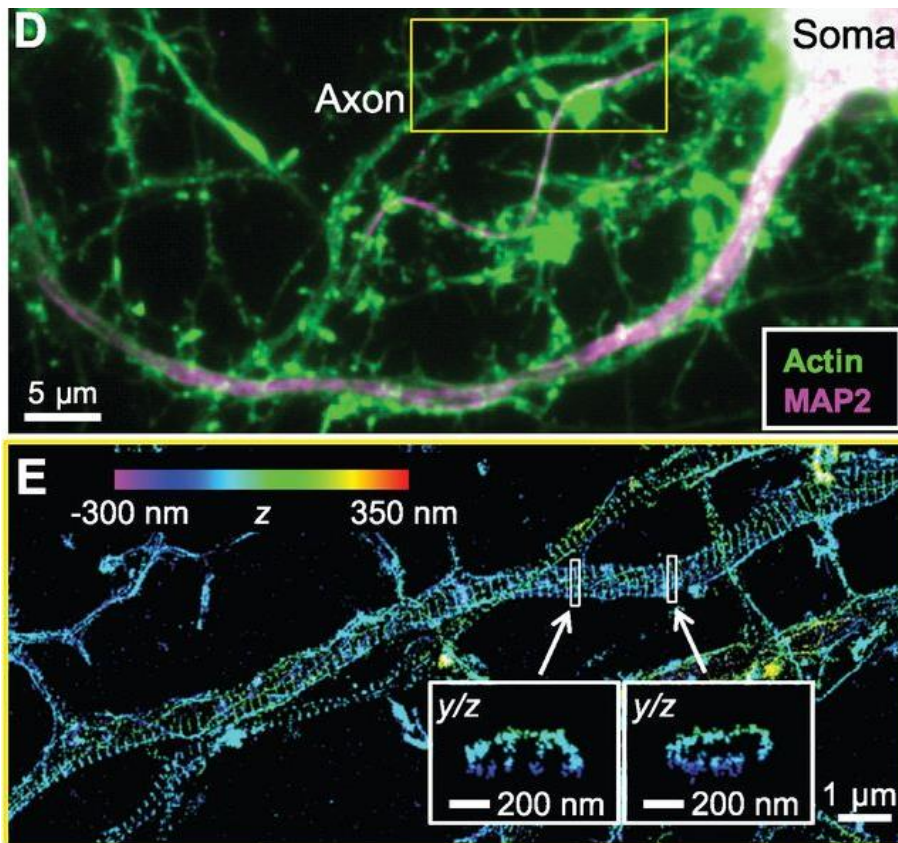
Actin filaments in axons form a quasi-1D, periodic structure with a uniform spacing of ~180 to 190 nm.
(**D**) Conventional fluorescence image of actin (green) and MAP2 (dendritic marker, magenta) in a neuron fixed at 12 DIV. (**E**) 3D STORM image of actin in a region containing axons (devoid of the MAP2). The **yz cross sections** corresponding to the white-boxed regions are shown. (A) Three-dimensional STORM image of a segment of axon (top) and the **distribution of localized molecules** after the 3D image was projected to one dimension along the axon long axis (bottom). (C) Histogram of the spacings between adjacent actin ringlike structures (N = 204 spacings). The red line is a Gaussian fit with a mean of 182 nm and a SD of 16 nm.

Laurent Blanchoin: NRS, BIG/DRF/CEA,
Cytomorpholab Grenoble
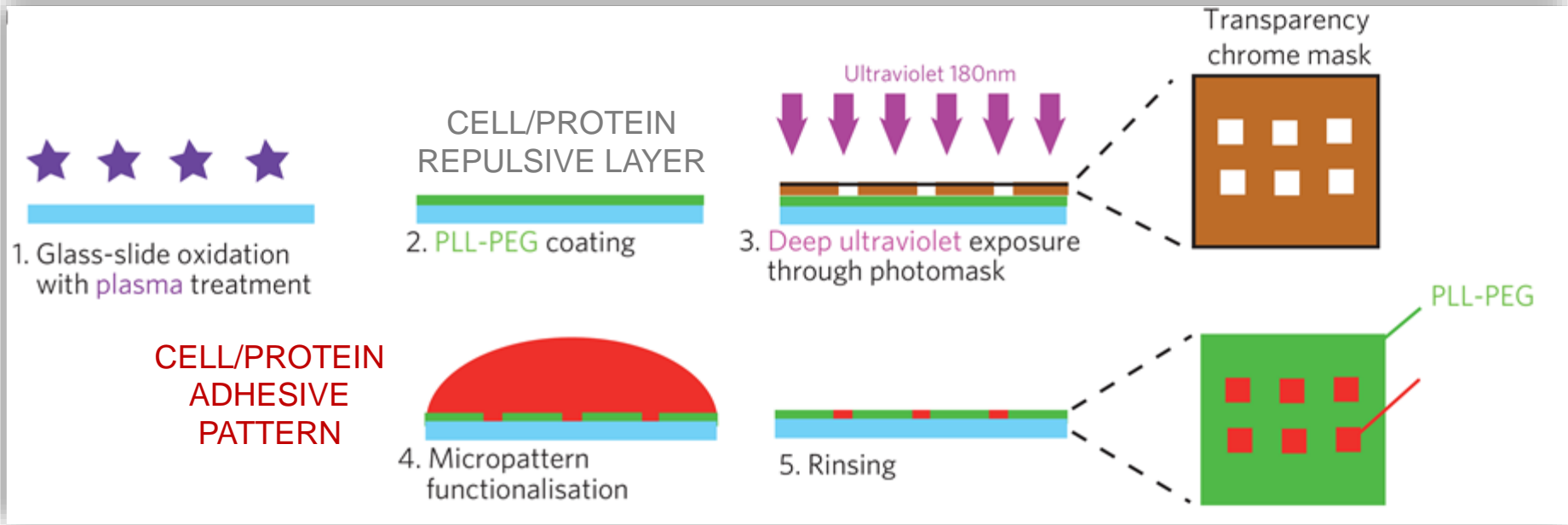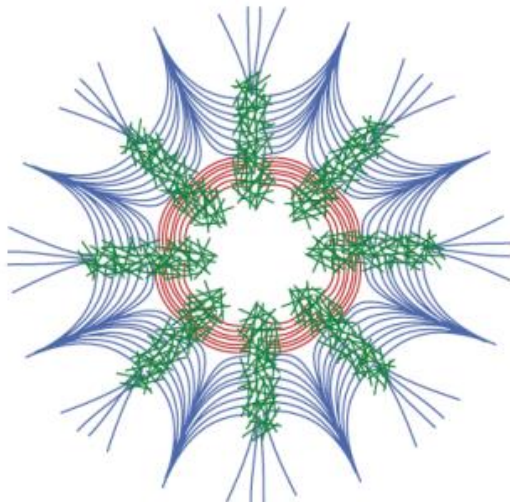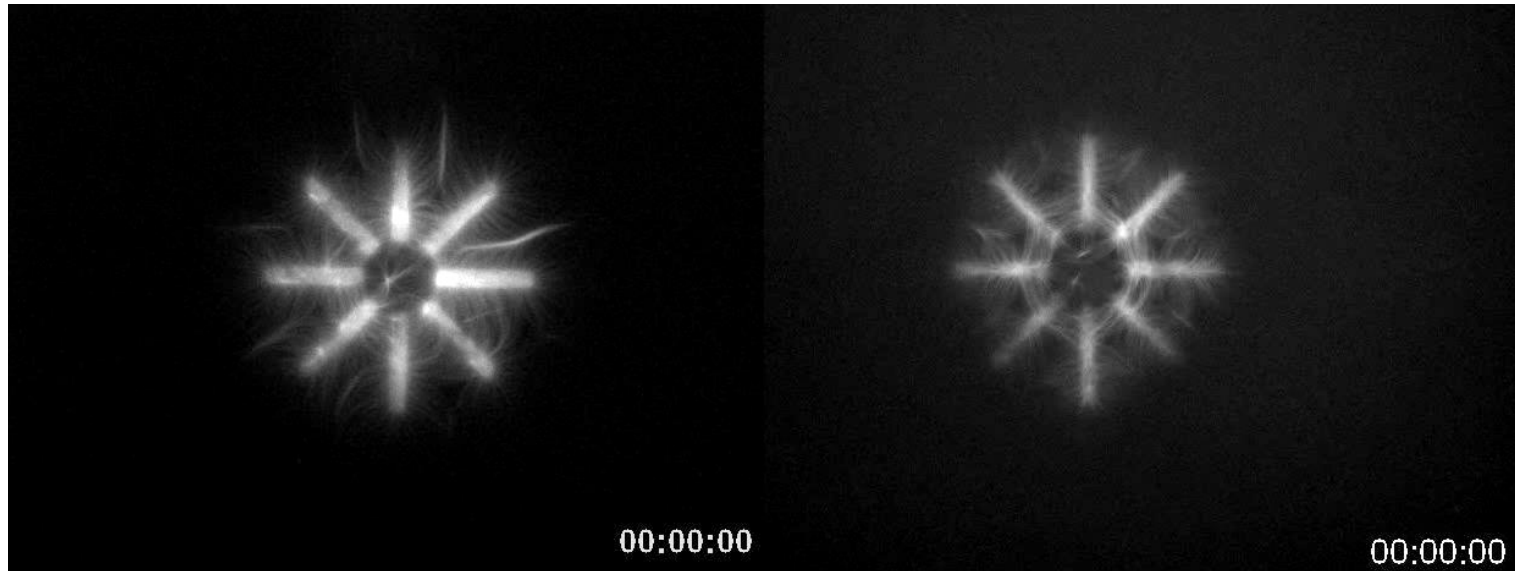
ANGLE (direction!)
BETWEEN FILAMENTS

Actin filaments :

anti-parallel

parallel

branched
meshwork

PARALLEL

BRANCHED

ANTIPARALLEL

Reyman AC Science 2012
Reymann AC. et al. Nature Materials 2010

PARALLEL

BRANCHED

ANTIPARALLEL

ANGLE (direction!)
BETWEEN FILAMENTS

Actin filaments :
anti-parallel
parallel
branched
meshwork
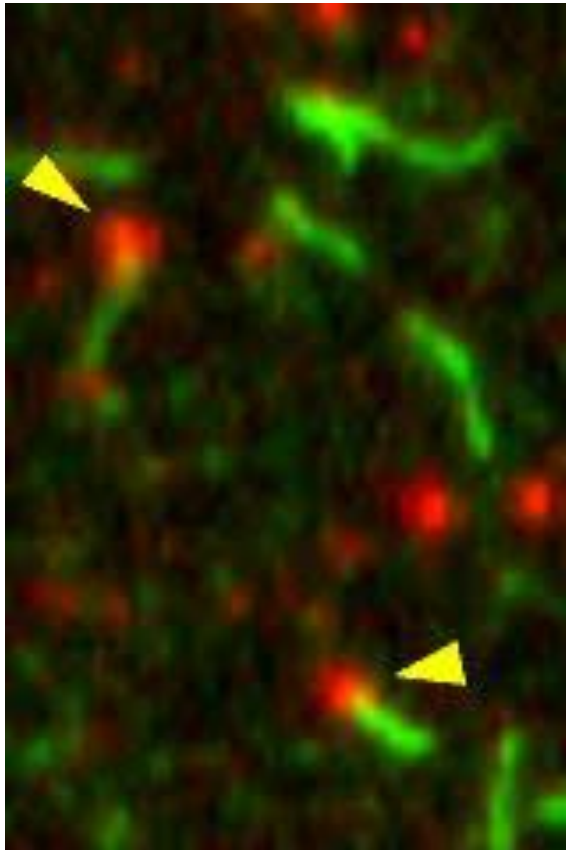
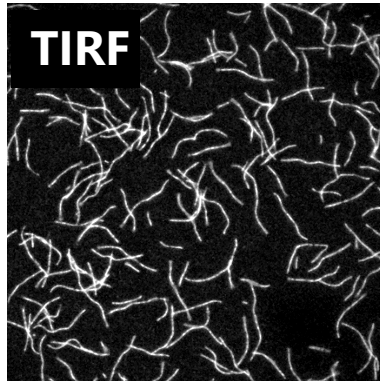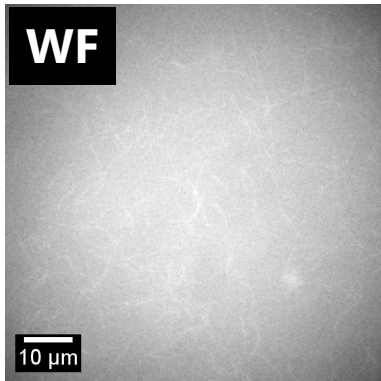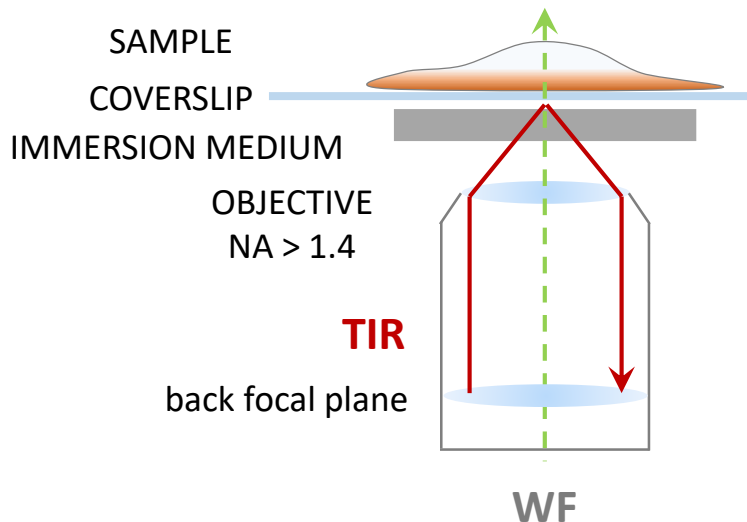18 nM QD-Bni1(FH1FH2)p_{bio} + 1.5 µM Actin
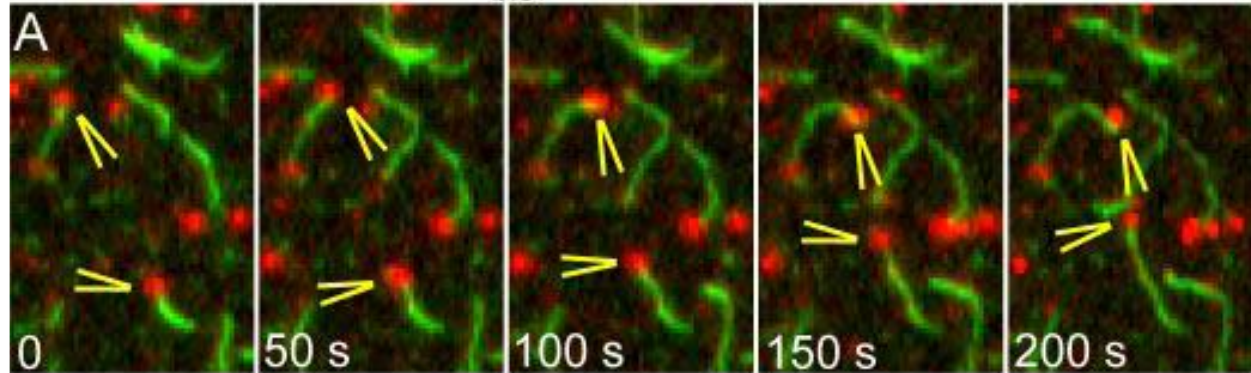
Courtesy: Paul A., Pollard T.C. JBC 2009

FORMIN: QDot 625 nanocrystal
ACTIN: Alexa488

STUDYING THE DYNAMIC BEHAVIOUR OF ACTIN
DIRECT „OBSERVATION" OF ABPs ACTIVITIES

WF

TIRF

10 μm

OBJECTIVE BASED TIRFM

SAMPLE
COVERSLIP
IMMERSION MEDIUM
OBJECTIVE NA > 1.4
TIR
back focal plane
WF
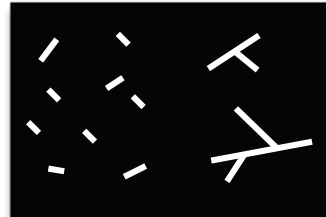
SPONTANEOUS ACTIN DYNAMICS
$k_+, k_-, [G], [F]$

NUCLEATION FACTORS
$[F], k_+, k_-$
e.g. formins, Arp2/3 complex

SEQUESTRATORS
$[G], [F]$
e.g. WH2 domain proteins

ELONGATION/CAPPING FACTORS
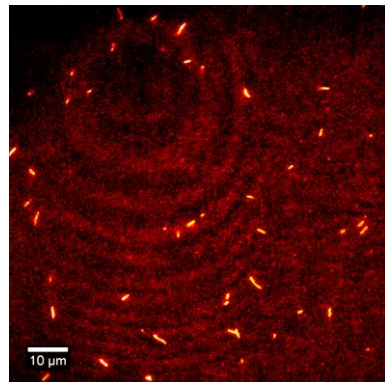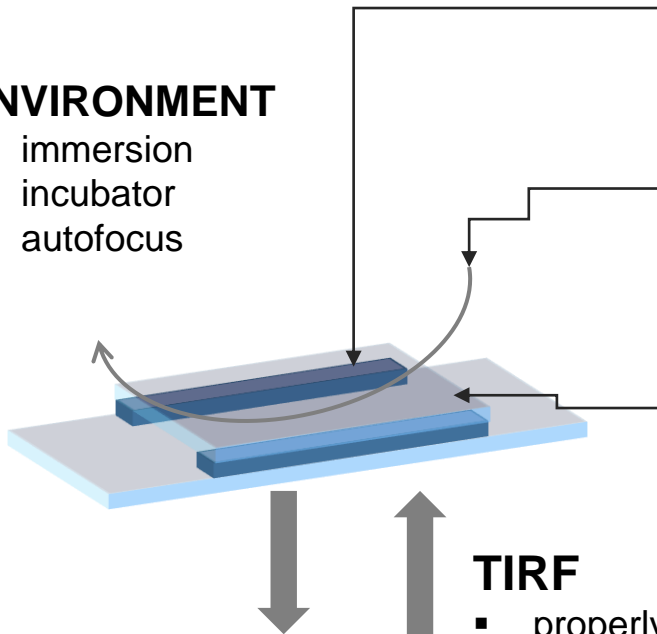k_+, k_-
e.g. formins, VASP, CP

FILAMENT BINDING
$k_+, k_-, [F]$
severing, stabilization

1001 arcú fehérjék: Fénymikroszkópiák a sejtszintű fehérjekutatásban (in Hungarian)

SAMPLE (cells, proteins, …)
- fluorescently labeled, no restriction of usable fluorphores (!available wavelength, and filter set)
- oxigen scavenger system
- low refractive index medium

FLOW CELL
- manual / microfluidics

SURFACE CHEMISTRY
- poly-L-lysine (PLL)
- collagen/fibronectin
- PLL-g-PEG
- streptavidine-biotin
- NEM-myosin

ENVIRONMENT
- immersion
- incubator
- autofocus

TIRF
- properly aligned laser beam
- no optical imperfections
- properly mounted coverslip (no tilt!)

- interference fringes
- inhomogeneous field

10 µm

Biotin
Streptavidin
PLL-g-PEG
PLL backbone
PEG side chains
Glass

PLL-PEG
Parafilm
Rinse and dry
3
UV 180 nm
UV 180 nm
Transparency chrome mask
4b Water
Vacuum
5 Fibronectin
Parafilm
6 PLL-PEG
Fibronectin

NEM MyosinII   **BSA**   **Actin Filaments/bundles**

Azioune et al. Lab on a Chip 2009
Reymann AC. et al. Nature Materials 2010