

Total Internal Reflection Fluorescence (TIRF) microscopy in cell biology.

QuickTime™ and a
Animation decompressor
are needed to see this picture.

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Total Internal Reflection Fluorescence (TIRF) microscopy in cell biology.

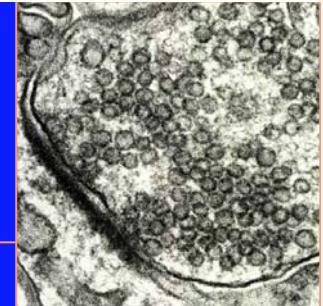
- Principles of the technique
- Configuration of the microscope
- Examples:
 - Vesicle trafficking and fusion
 - Analysis
 - Structure of receptors
 - Endocytosis
 - Intracellular signaling
- Advantages disadvantages

Total Internal Reflection Fluorescence (TIRF) microscopy in cell biology.

Optical technique that restricts the excitation and detection of a fluorophores to a thin region of the specimen.

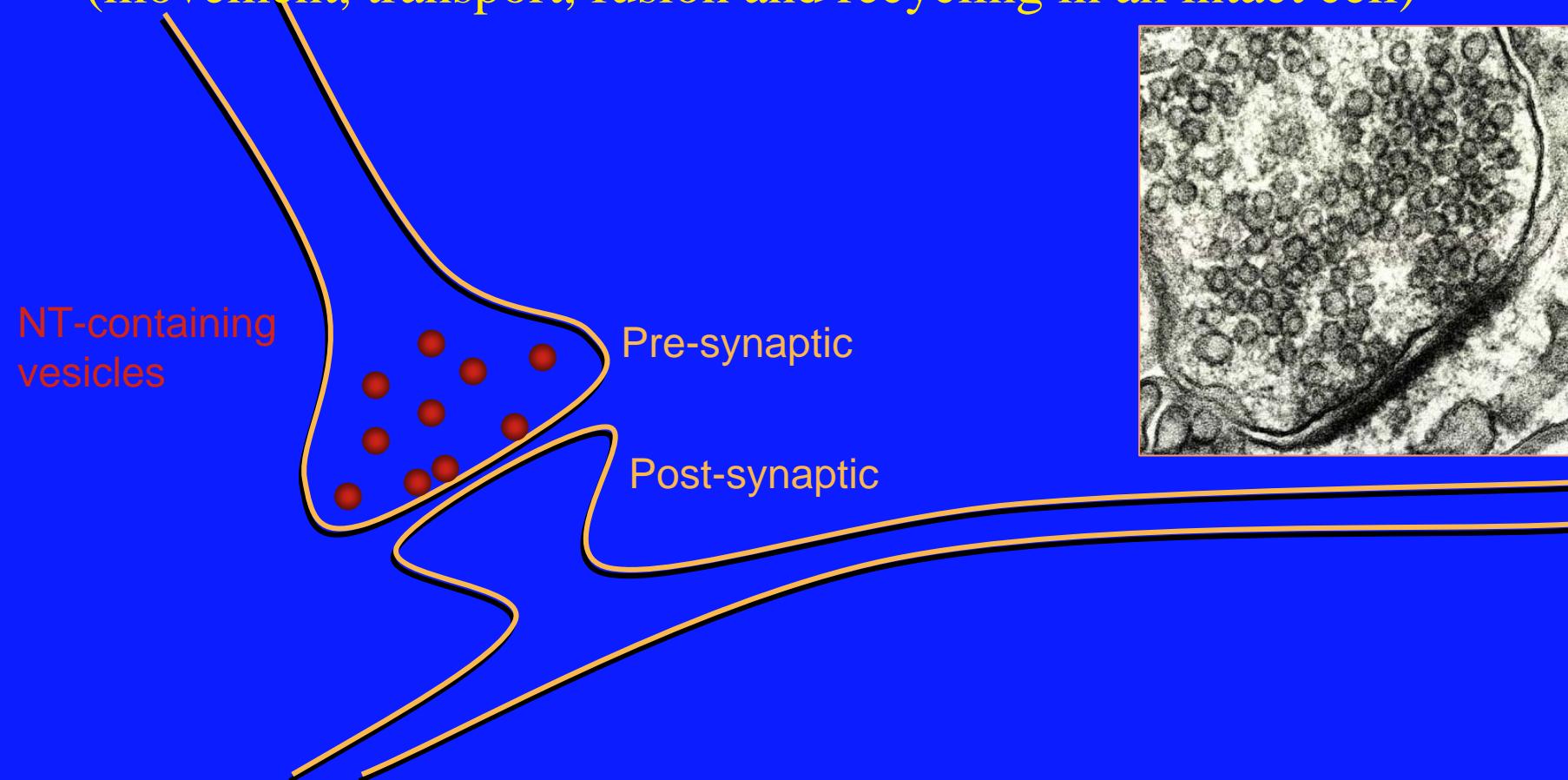
- Allows direct visualization and detection of sub-membrane events:
 - movement and fusion of single vesicles
 - conformational dynamics of single ion channels and receptors
 - translocation of proteins to and from the plasma membrane
 - protein-protein interaction at the plasma membrane
 - cell adhesion processes

Synaptic transmission is carried out by fusion of neurotransmitter-containing vesicles

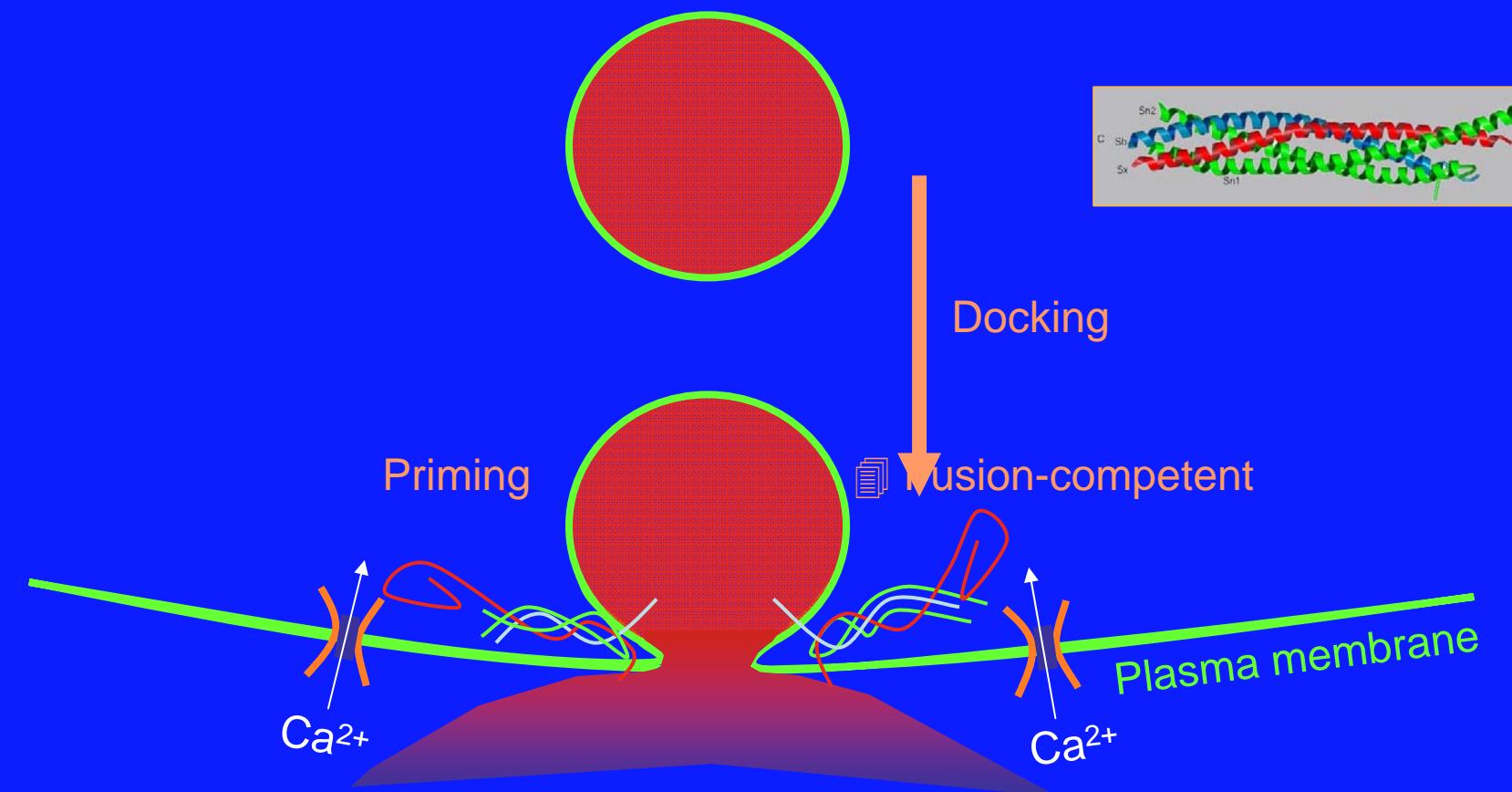
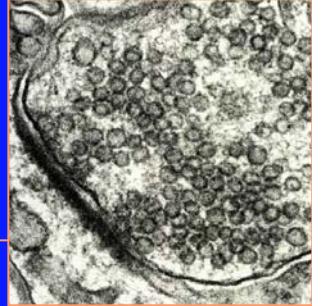


Molecular mechanisms of vesicle exocytosis and endocytosis:

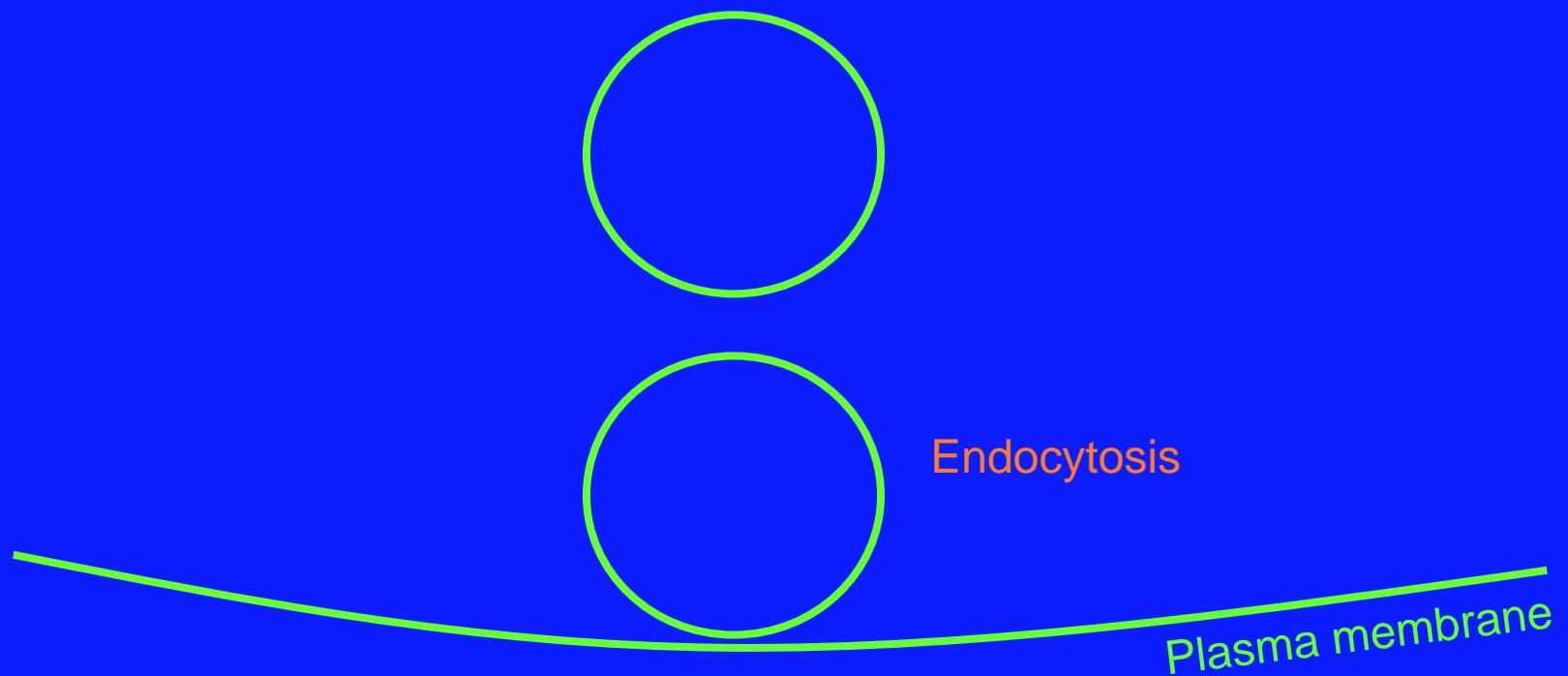
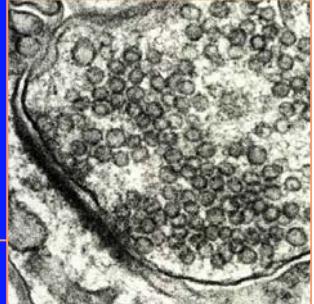
TIRF allows direct visualization of single vesicles
(movement, transport, fusion and recycling in an intact cell)



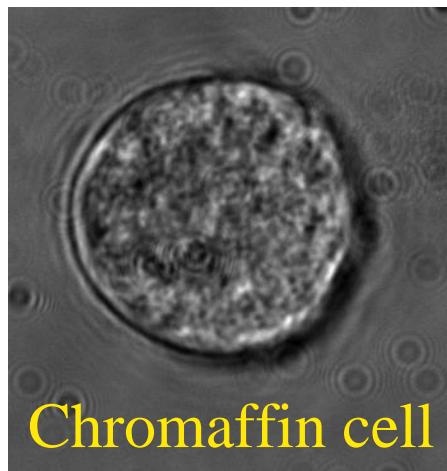
The synaptic vesicle cycle



The synaptic vesicle cycle

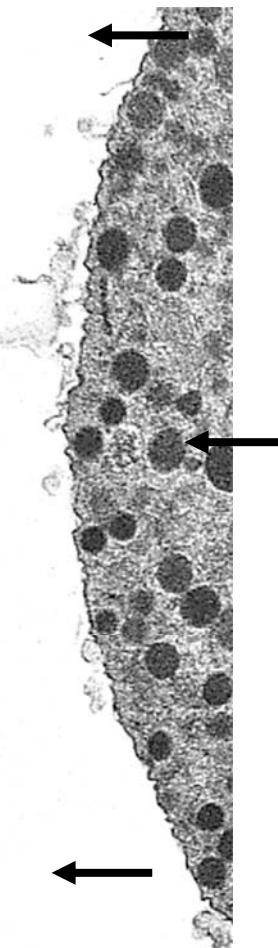
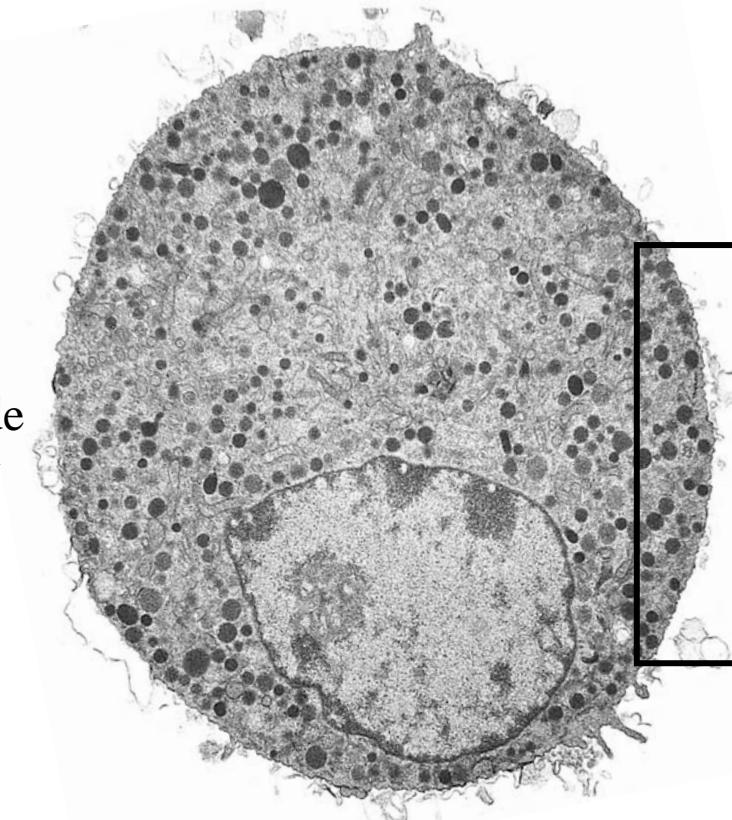


The chromaffin cell - a model system for neuro-exocytosis: Chromaffin cells secrete adrenaline into the blood stream



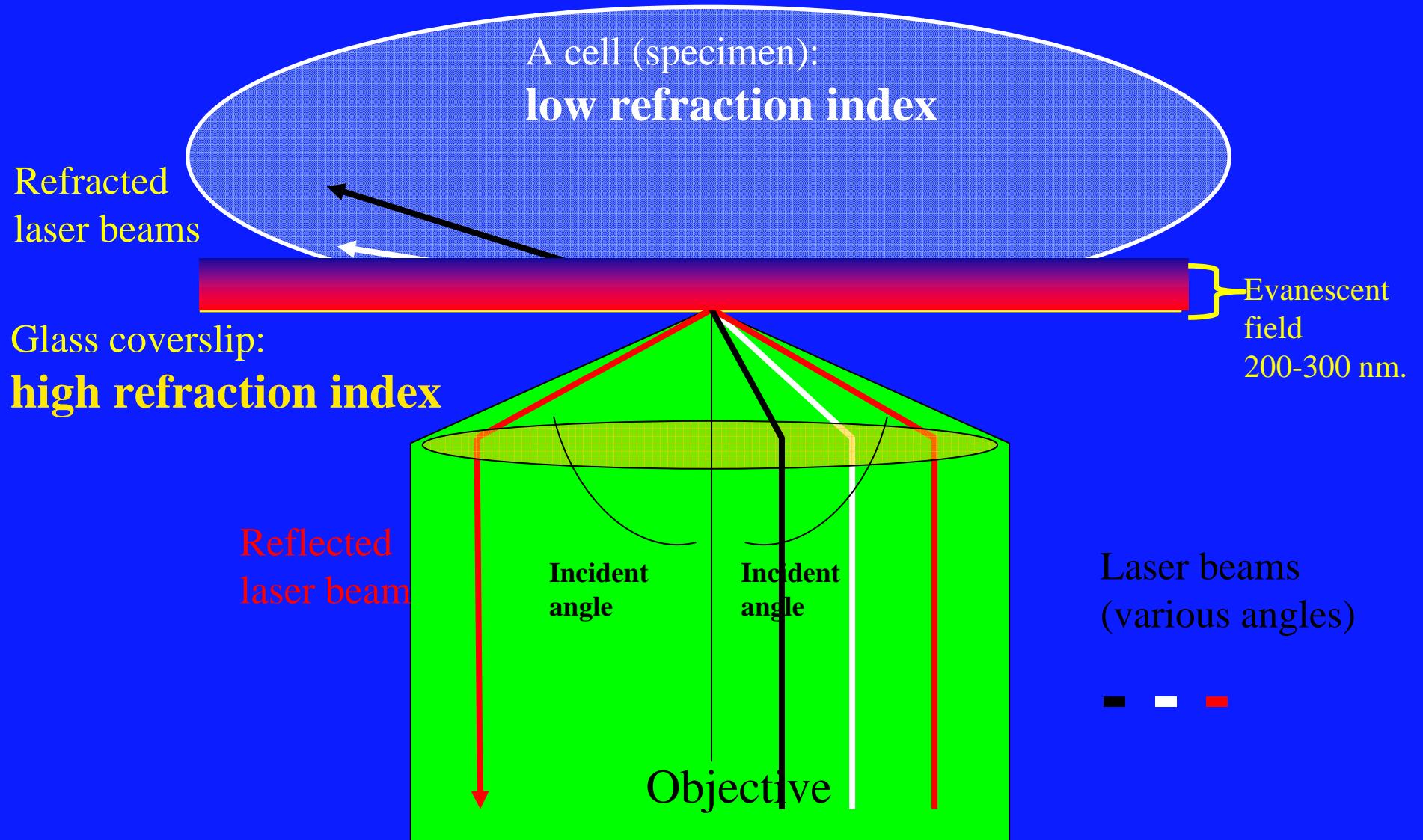
Chromaffin cell

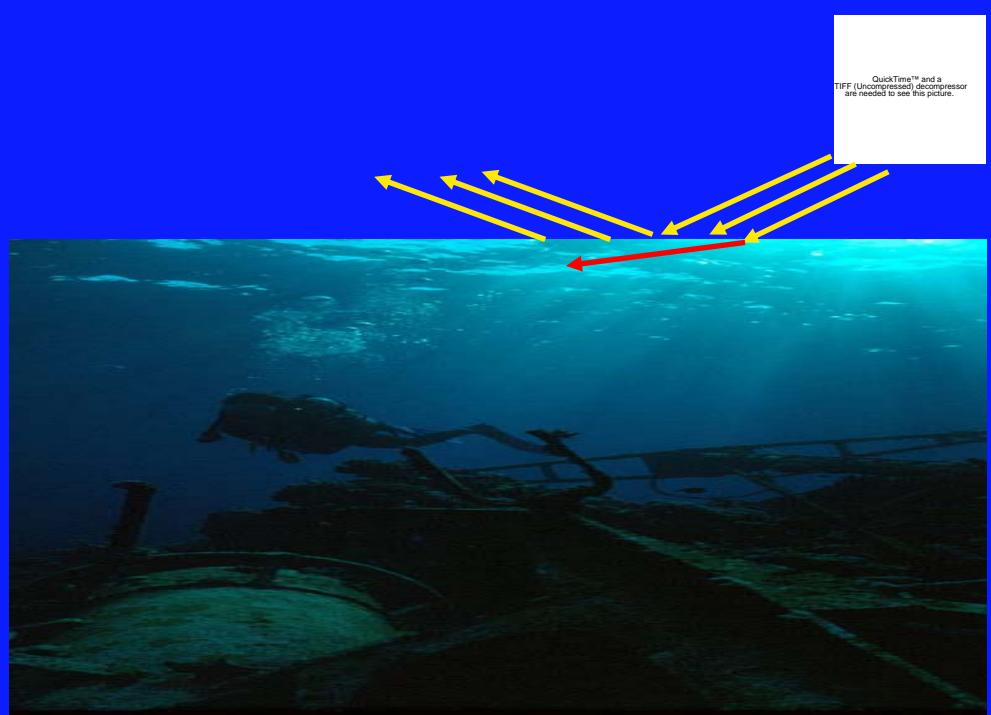
What's inside
→



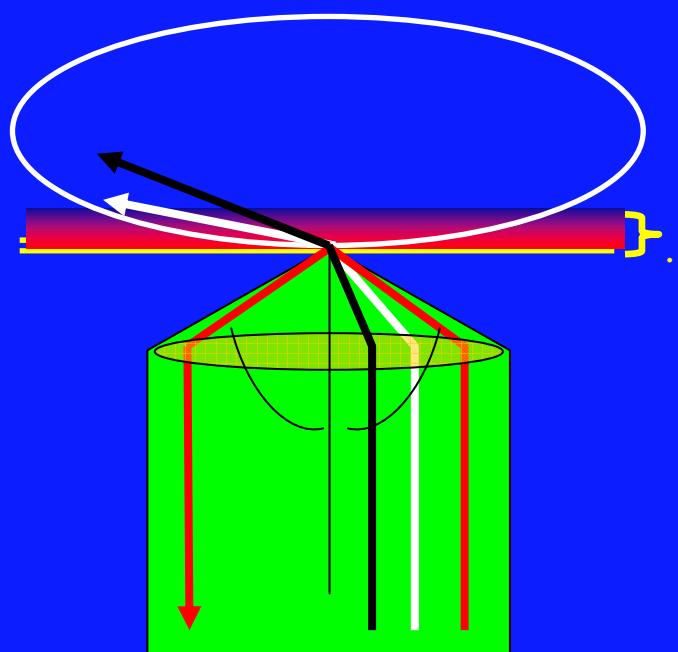
1μm

Total Internal Reflection Fluorescence (TIRF) or Evanescence-Wave Fluorescence Microscopy

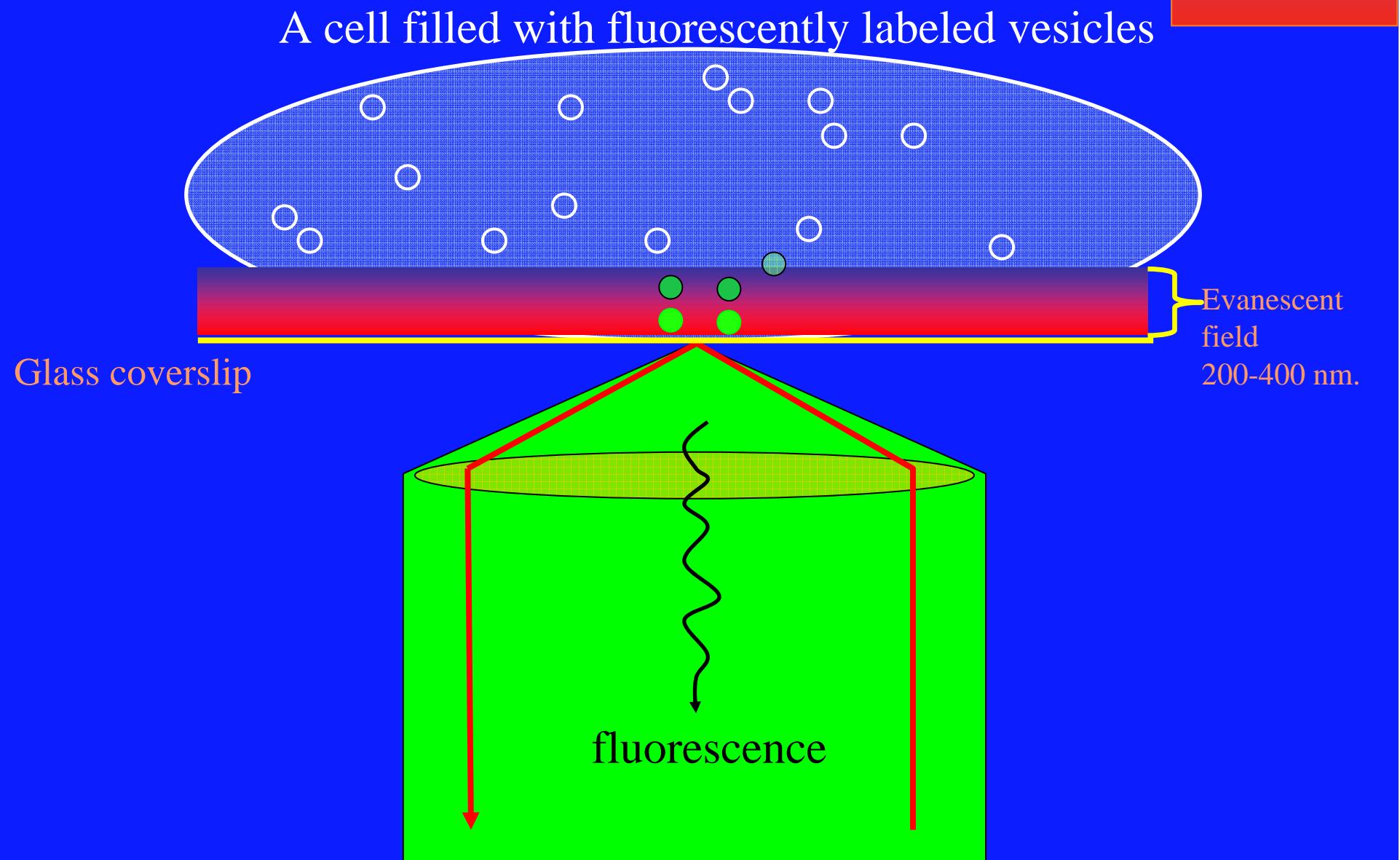


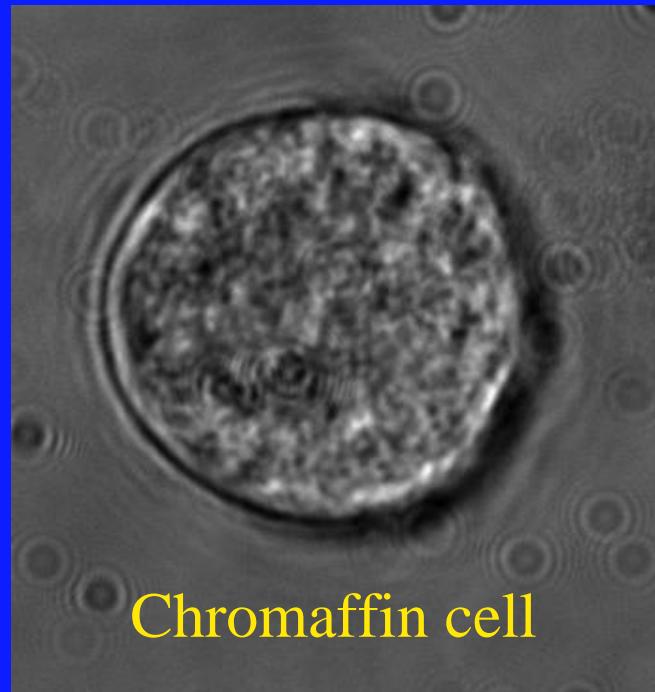


QuickTime™ and a
TIFF (Uncompressed) decompressor
are needed to see this picture.

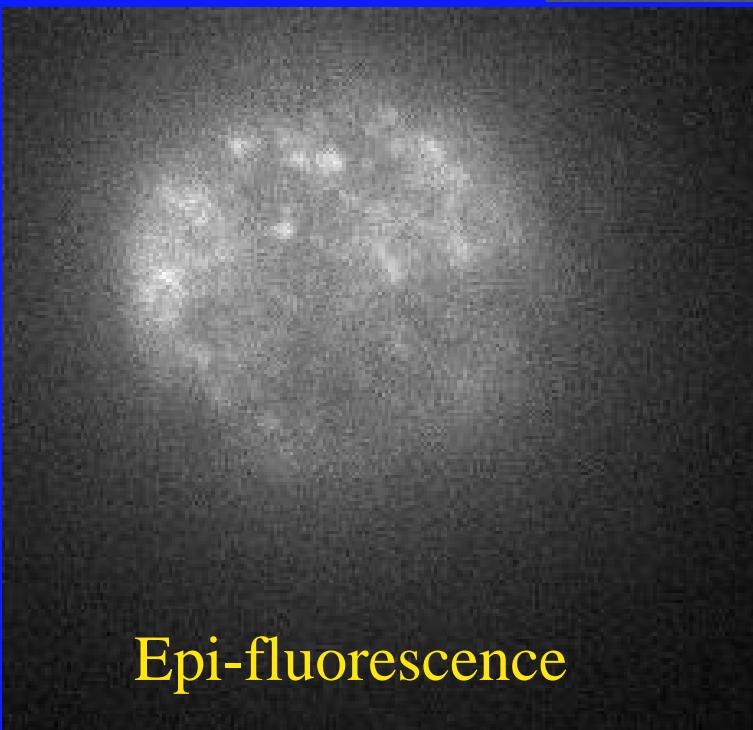


Total Internal Reflection Fluorescence (TIRF) or Evanescent-Wave Fluorescence Microscopy

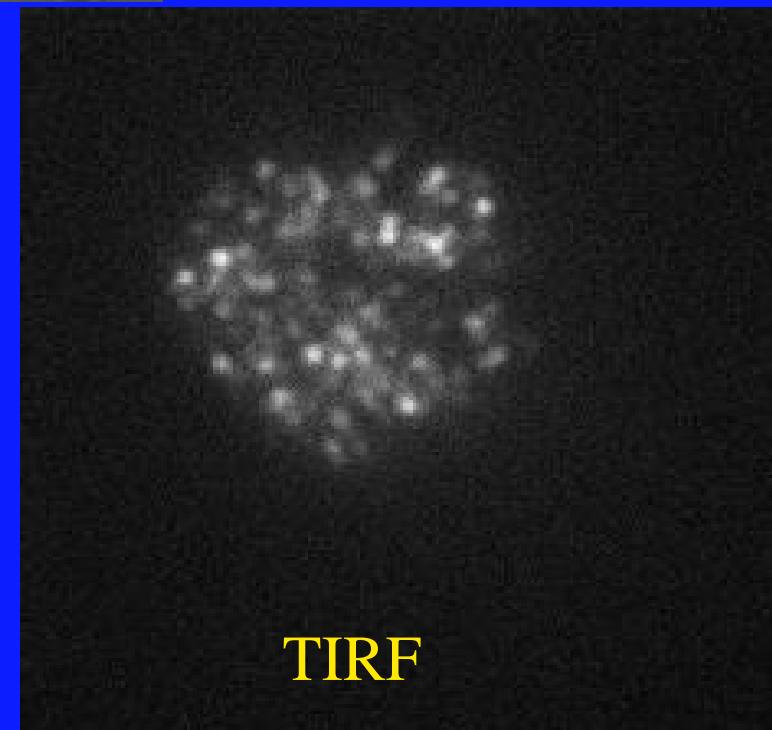




Chromaffin cell

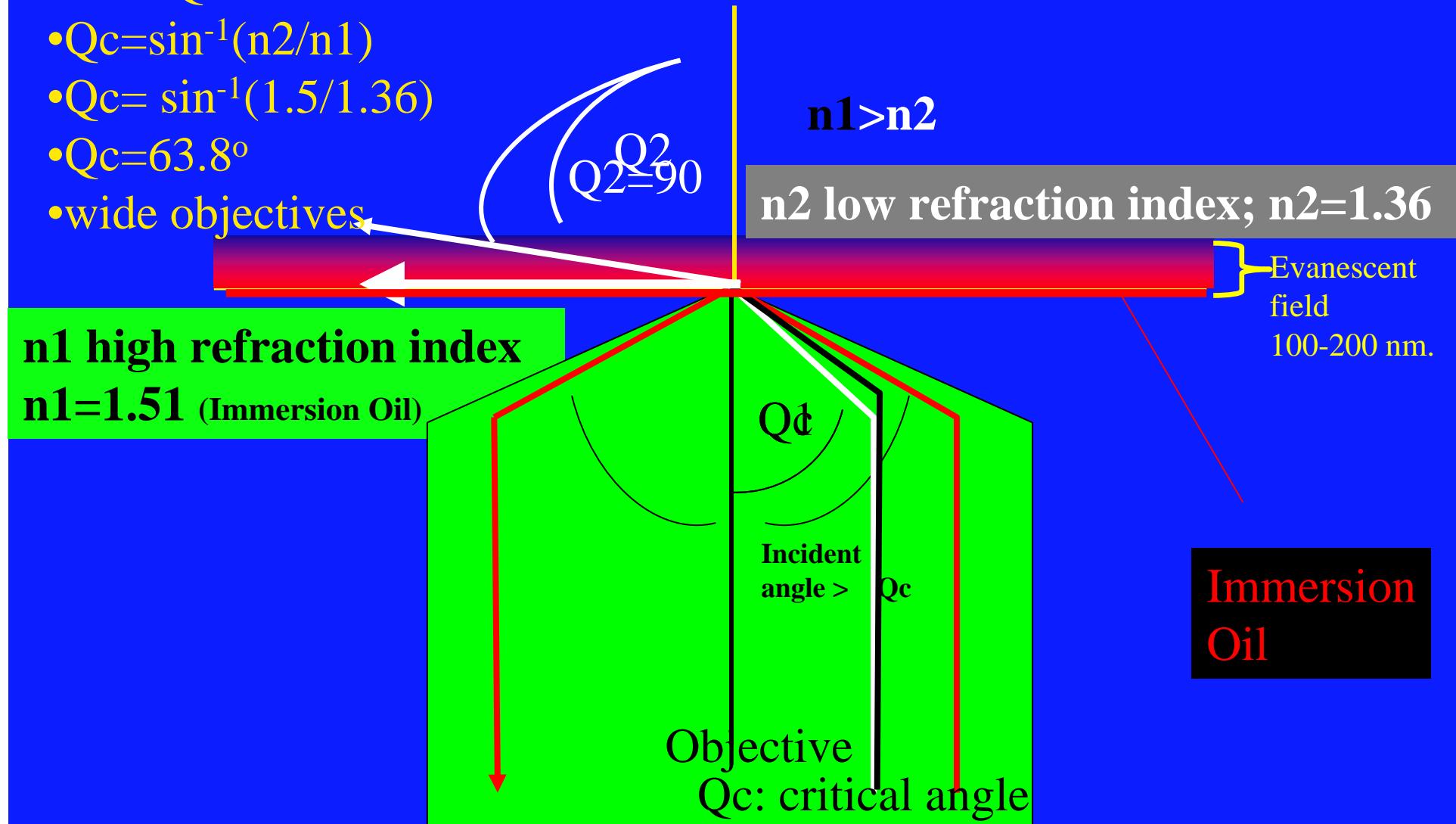


Epi-fluorescence



TIRF

- Snell's law: $n_1 \sin Q_1 = n_2 \sin Q_2$
- Critical angle Q_c (Q_1), $Q_2=90^\circ$ and ($\sin 90^\circ=1$)
- $n_1 \sin Q_c = n_2$
- $Q_c = \sin^{-1}(n_2/n_1)$
- $Q_c = \sin^{-1}(1.5/1.36)$
- $Q_c = 63.8^\circ$
- wide objectives



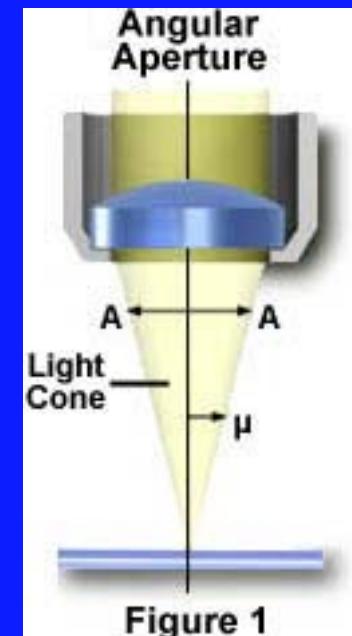
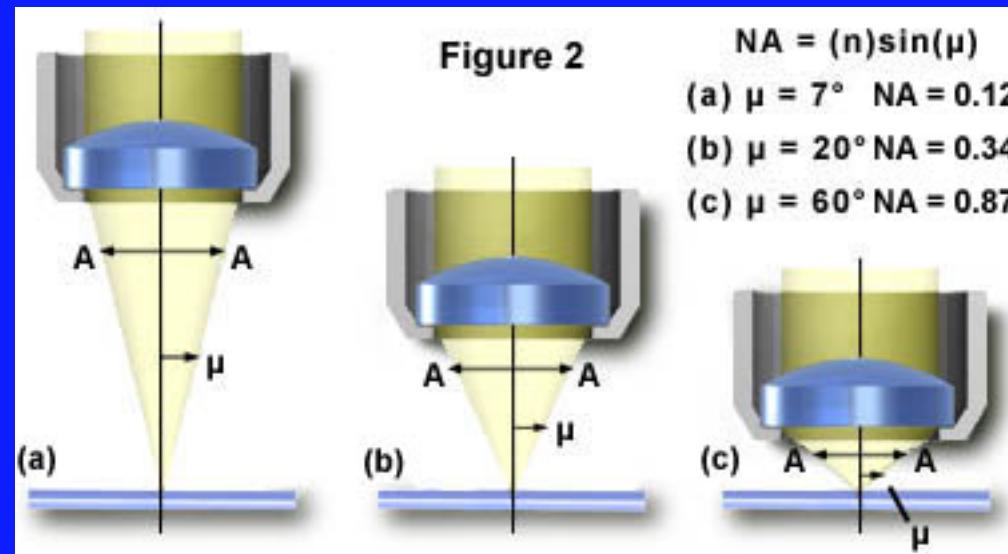
Air $n=1.00$, water $n=1.33$, Cytosol $n=1.36-1.38$, Immersion oil $n=1.51$

Thus Objective NA must be > 1.4 ; NA=1.45; one uses 10% of TIRF

The numerical aperture of a microscope objective is a measure of its ability to gather light and resolve fine specimen detail at a fixed object distance.

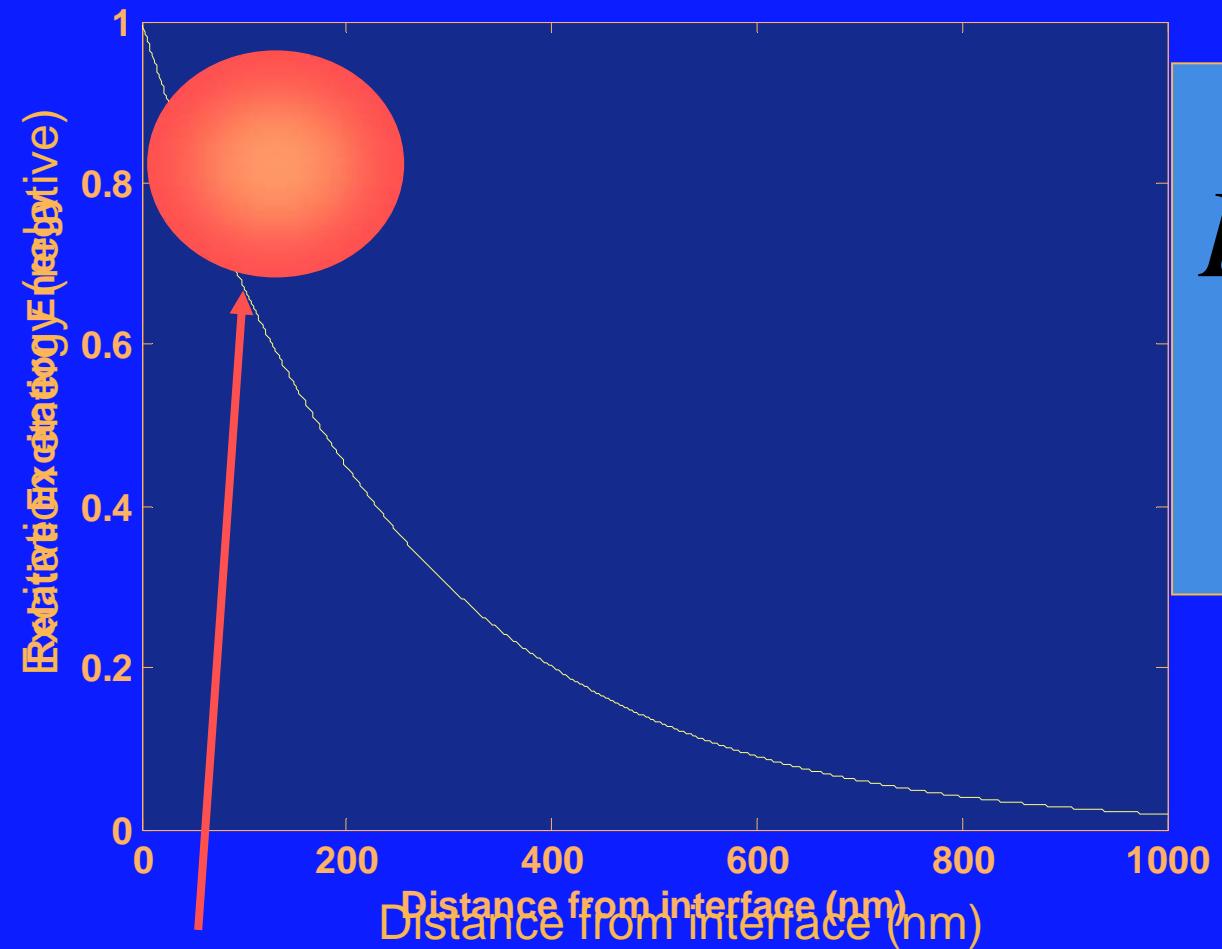
Numerical Aperture (NA) = $n(\sin m)$

n is the refractive index of the imaging medium
 $m=A/2$



Decay of the evanescence field

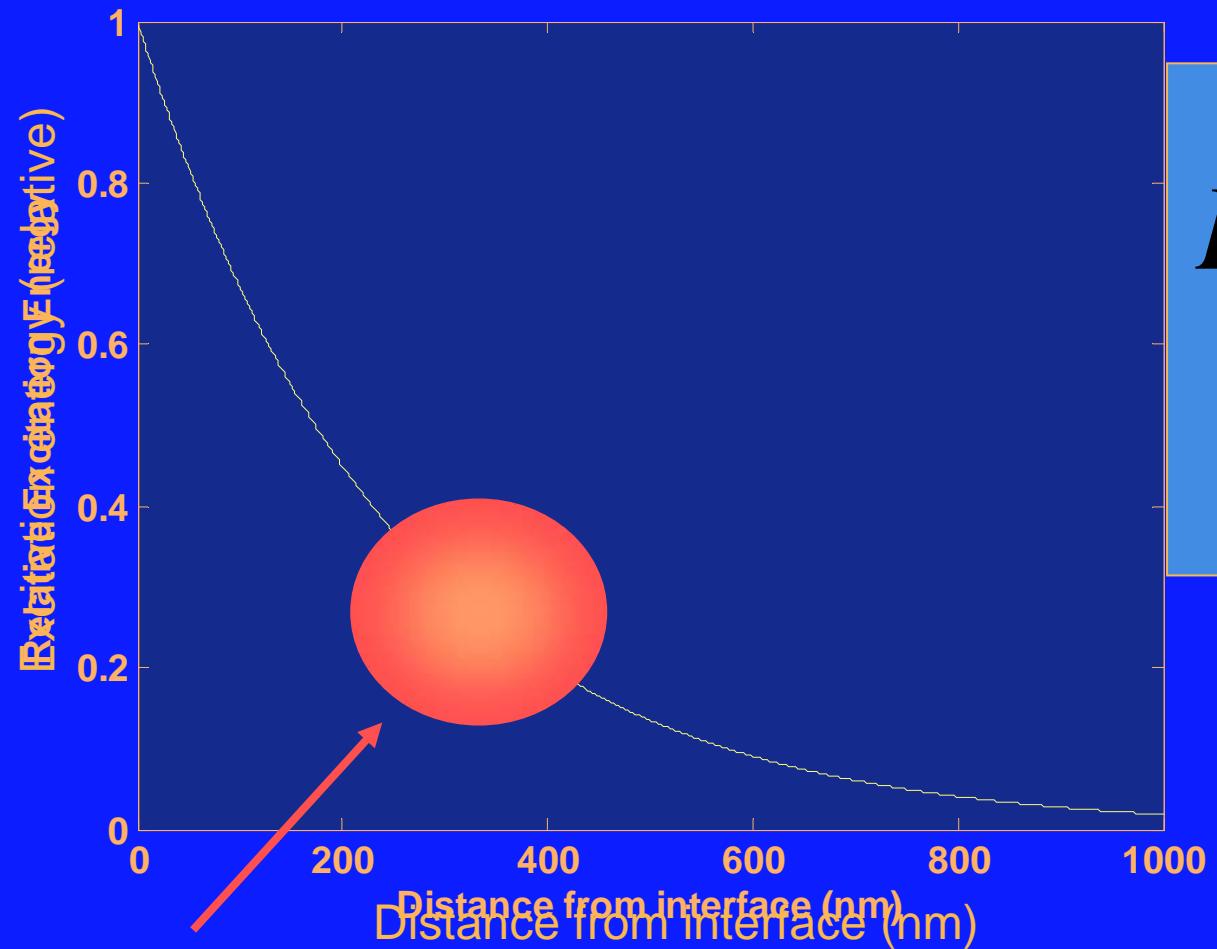
Evanescence field
100-200 nm.



$$E = C \times e^{-\frac{z}{\tau}}$$

C = Constant
z = Distance from membrane
 τ = Extinction coefficient
(penetration depth of the evanescence field)

Decay of the evanescence field

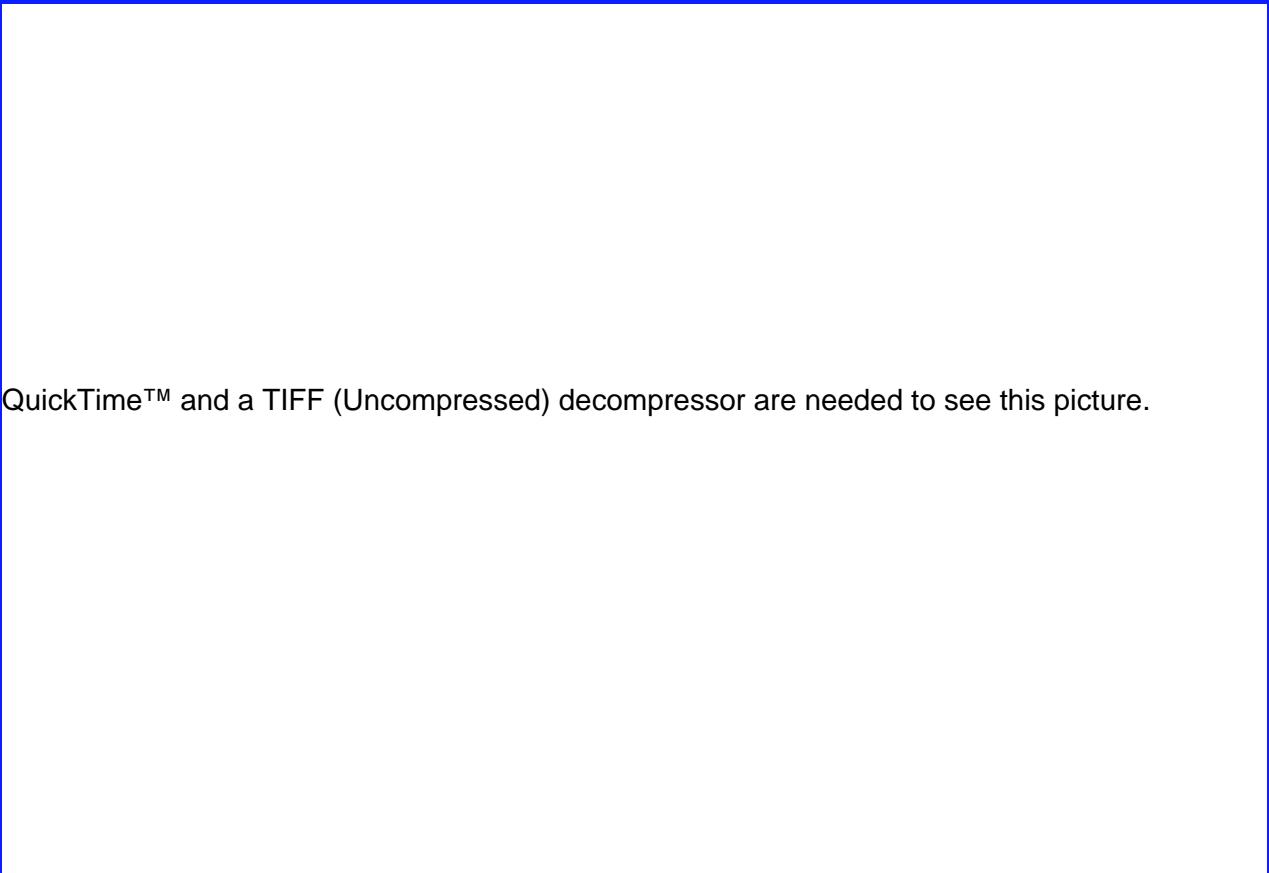


$$E = C \times e^{-\frac{z}{\tau}}$$

C = Constant
z = Distance from membrane
 τ = Extinction coefficient

Chromaffin vesicle

<http://www.micro.magnet.fsu.edu/primer/java/tirf/penetration/index.html>



QuickTime™ and a TIFF (Uncompressed) decompressor are needed to see this picture.

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

$$d = \lambda/4 \cdot (n(1) 2\sin 2q(1) - n(2) 2)^{-1/2}$$

Have a look at this web address for more details

<http://www.micro.magnet.fsu.edu/primer>

Types of TIRF microscopes

• Total Internal Reflection Fluorescence (TIRF) microscopy

• TIRF microscopy is a type of fluorescence microscopy.

• It uses evanescent wave excitation to image fluorescent molecules near a surface.

• The excitation light source is positioned at an angle greater than the critical angle of the interface between the sample and the immersion medium.

• This results in total internal reflection of the excitation light at the interface.

• The evanescent field, which decays exponentially with distance from the interface, excites fluorescent molecules within a thin layer near the surface.

• The emitted fluorescence is collected by an objective lens and imaged.

• TIRF microscopy provides high resolution images of fluorescent molecules near surfaces.

• It is often used to study protein-protein interactions at surfaces or to image individual molecules.

• TIRF microscopy can also be used to study the dynamics of fluorescently labeled molecules near surfaces.

• It has applications in fields such as cell biology, materials science, and nanotechnology.

• TIRF microscopy is a powerful tool for studying the behavior of molecules at surfaces.

• It can provide insights into the mechanisms of various biological processes and physical phenomena.

• TIRF microscopy is a valuable technique for advancing our understanding of the world around us.

• It is a testament to the power of science and technology to explore and discover.

• TIRF microscopy is a remarkable example of how we can use our knowledge and resources to better understand the world.

• It is a reminder that there is still much to learn and explore.

• TIRF microscopy is a wonderful example of the beauty and complexity of the natural world.

• It is a reminder that we are still learning and discovering.

• TIRF microscopy is a wonderful example of the power of science and technology to explore and discover.

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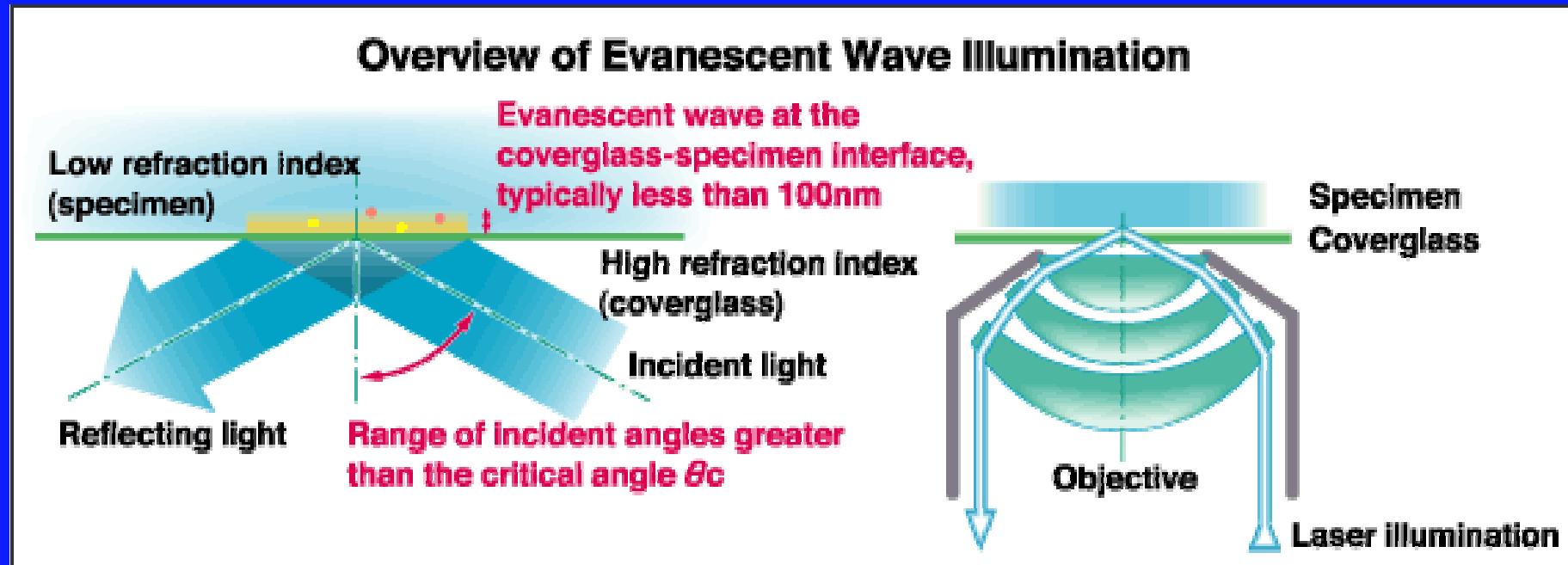
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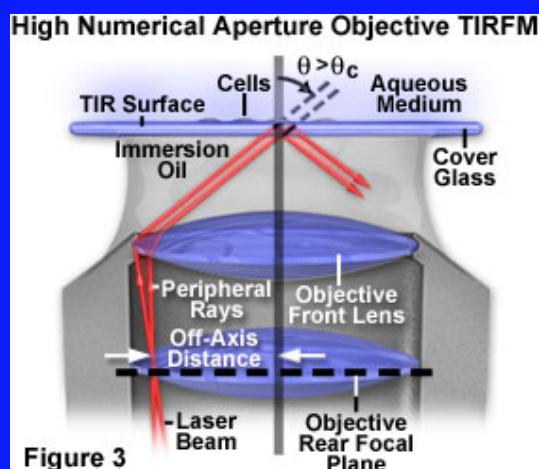
Objective type TIRF; Prism less (inverted microscope)

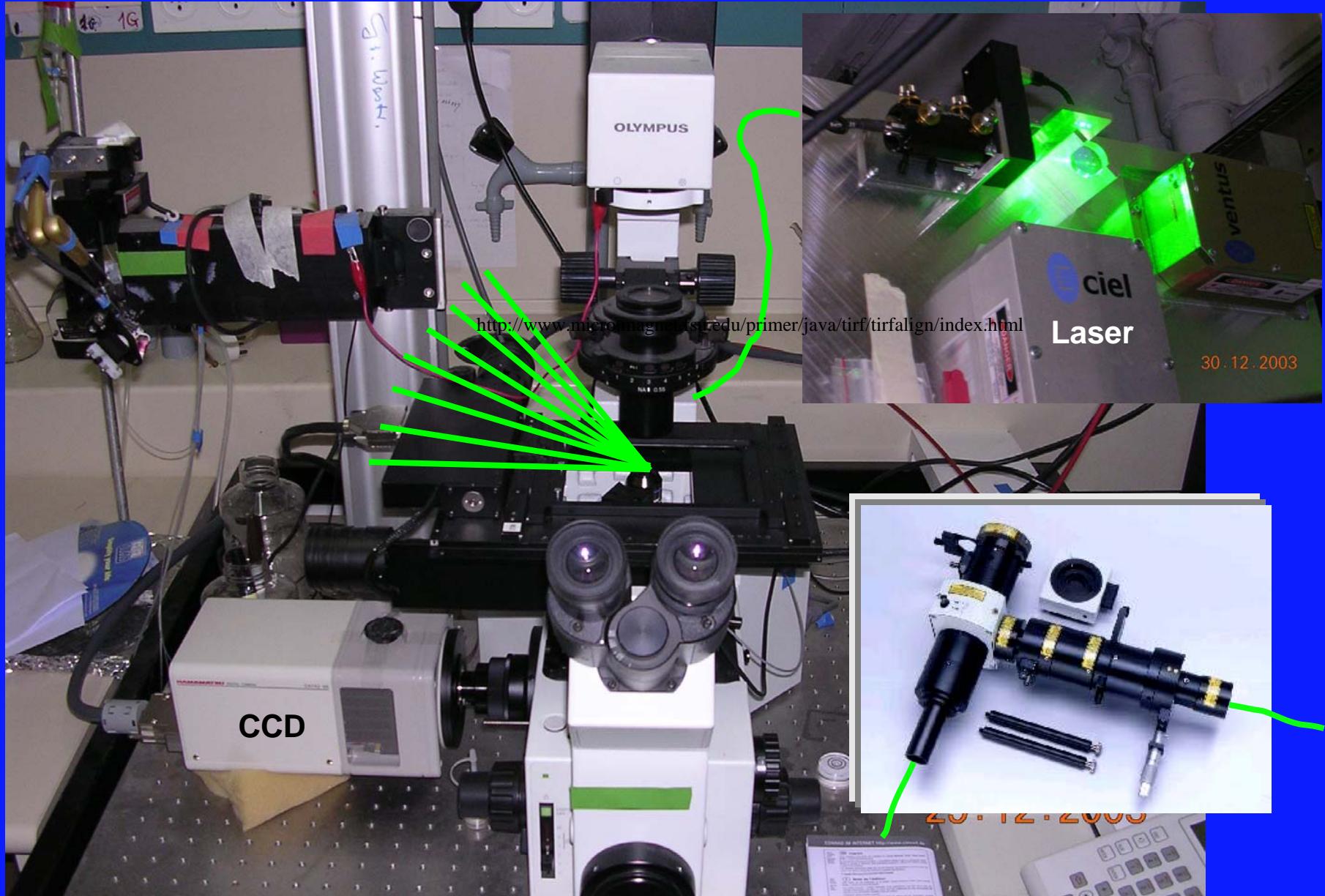


Objectives:

APO100XOHR
(NA 1.65)

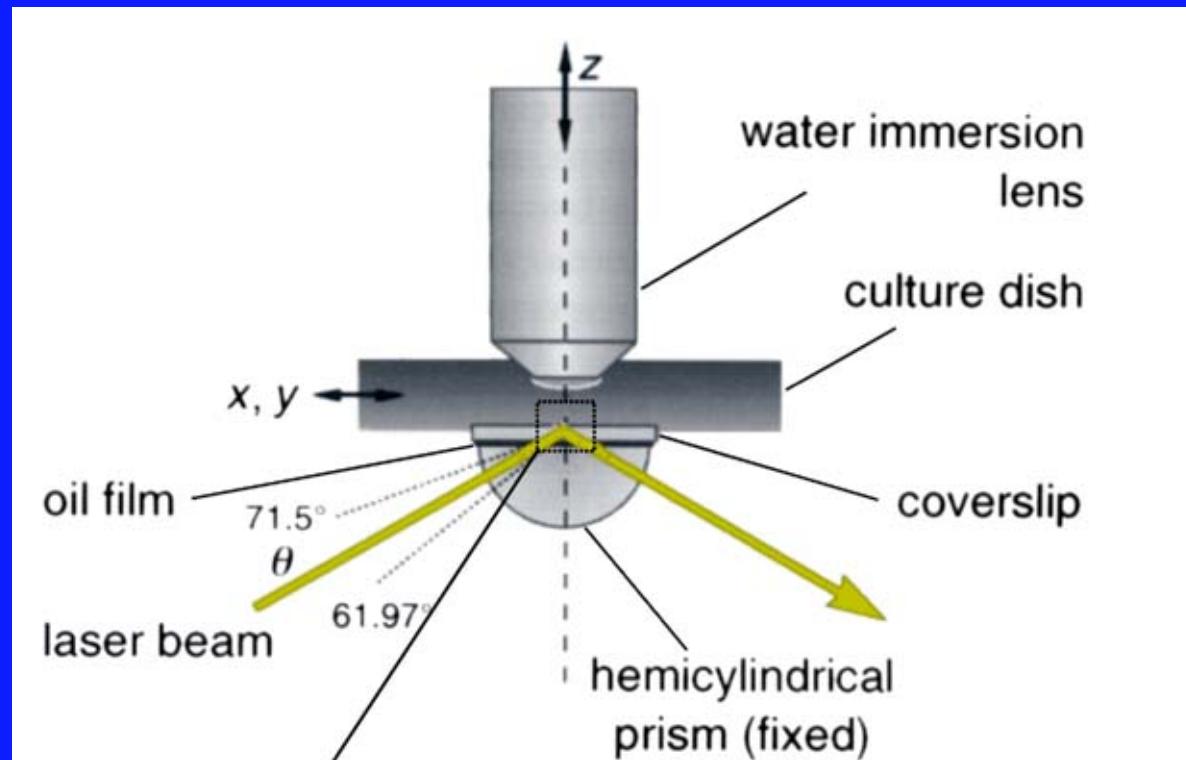
PLAPO60XOTIRFM
(NA1.45)





<http://www.micro.magnet.fsu.edu/primer/java/tirf/tirfalign/index.html>

Prism type TIRF (upright microscope)



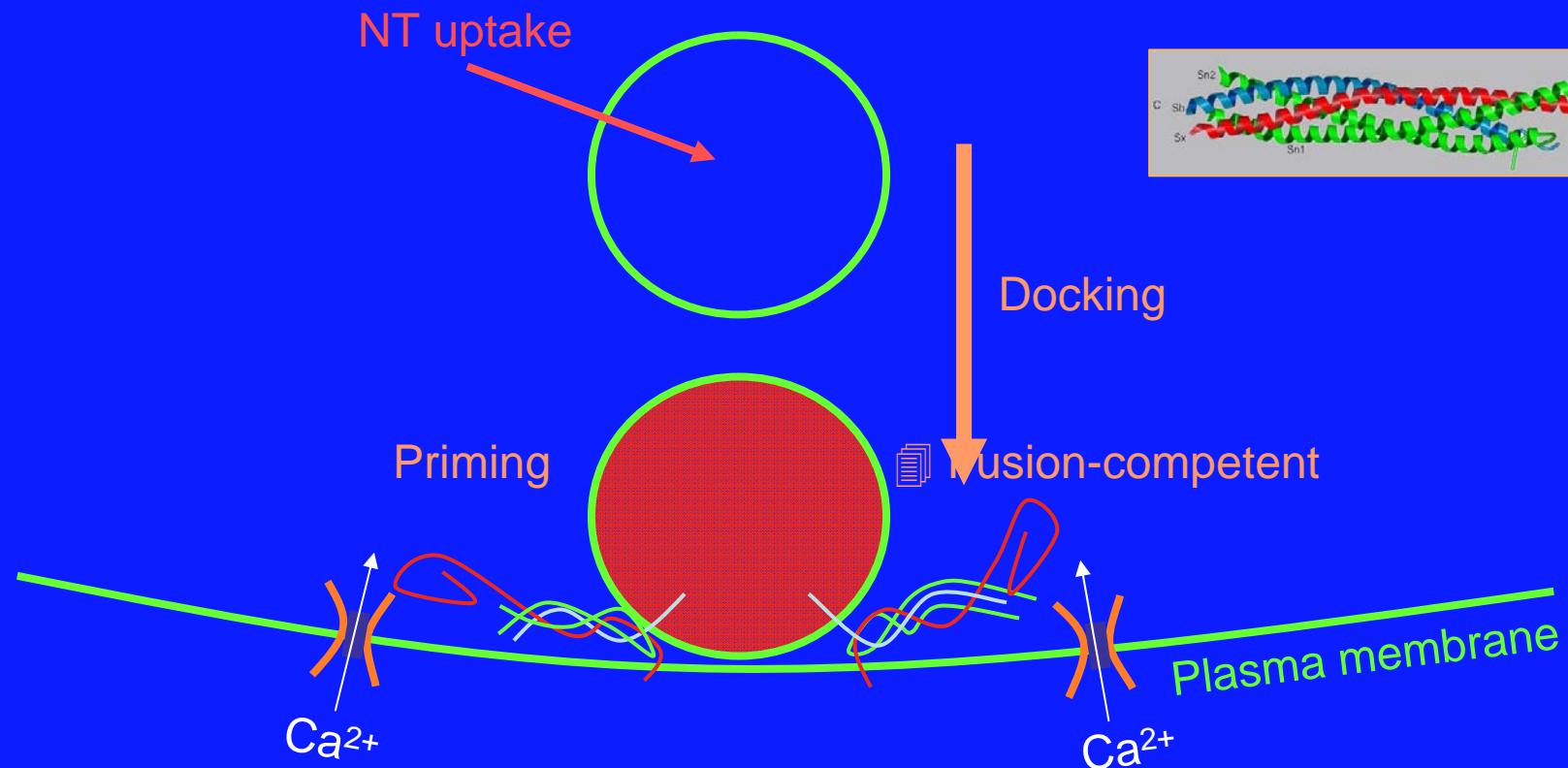
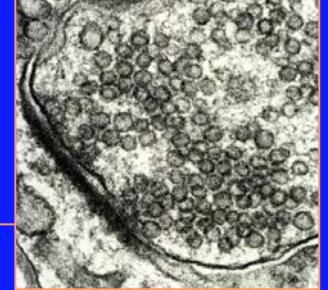
Benefit of the prism TIRFM

By varying the angle of the laser beam, one can modulates the penetration depth.

- Examples:
 - Vesicle trafficking and fusion
 - Structure of receptors
 - Endocytosis
 - Cellular signaling

Evanescence-Wave Fluorescence Microscopy

The synaptic vesicle cycle

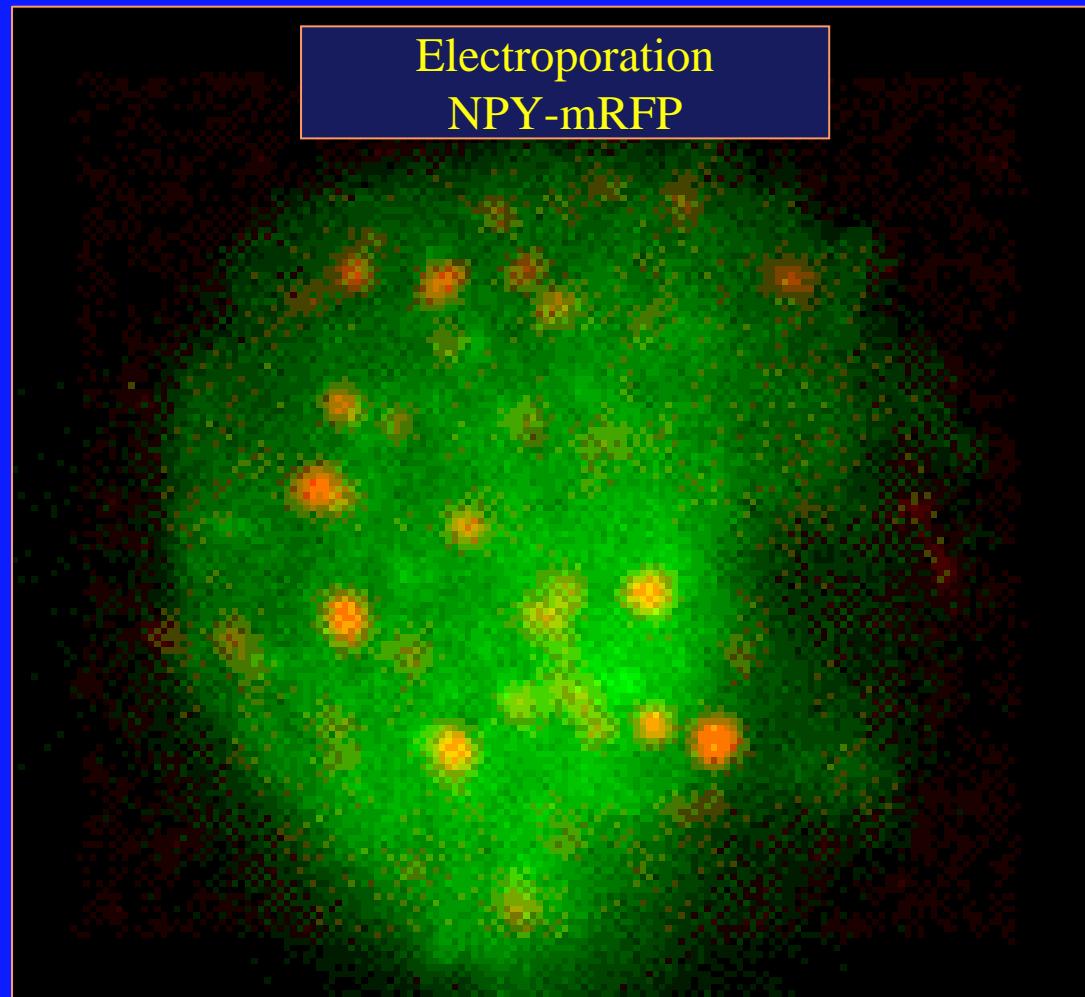


Allows direct visualization of number of docked vesicles, fraction of fusion-competent vesicles and vesicles transport in an intact cell.

Staining chromaffin vesicles with fluorescent proteins

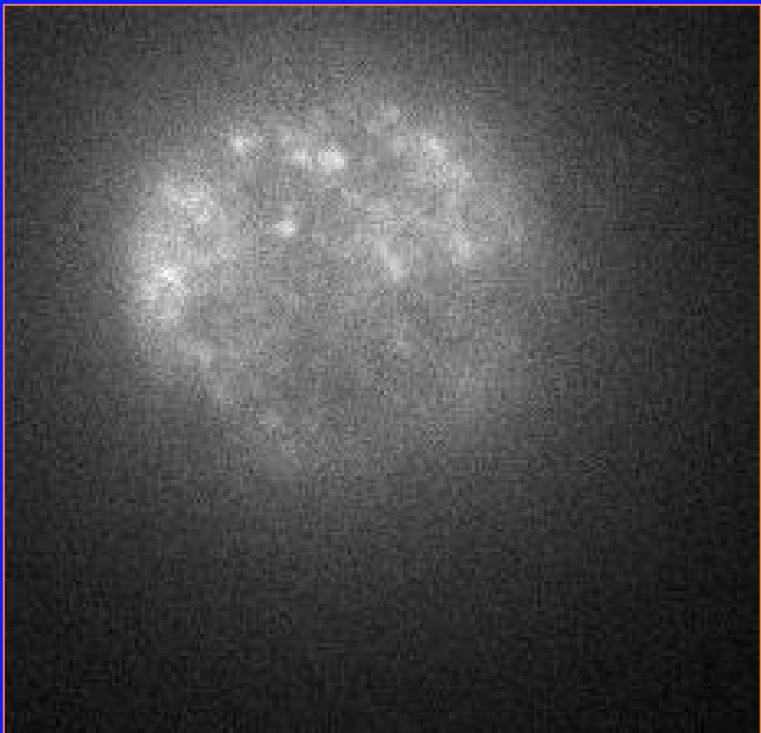
- Acidic dyes (Acridine Orange, Lysotracker)
- Vesicular proteins:
 - Membrane
 - Lumen

Endocytotic dyes



What's it good for?

Epi-fluorescence



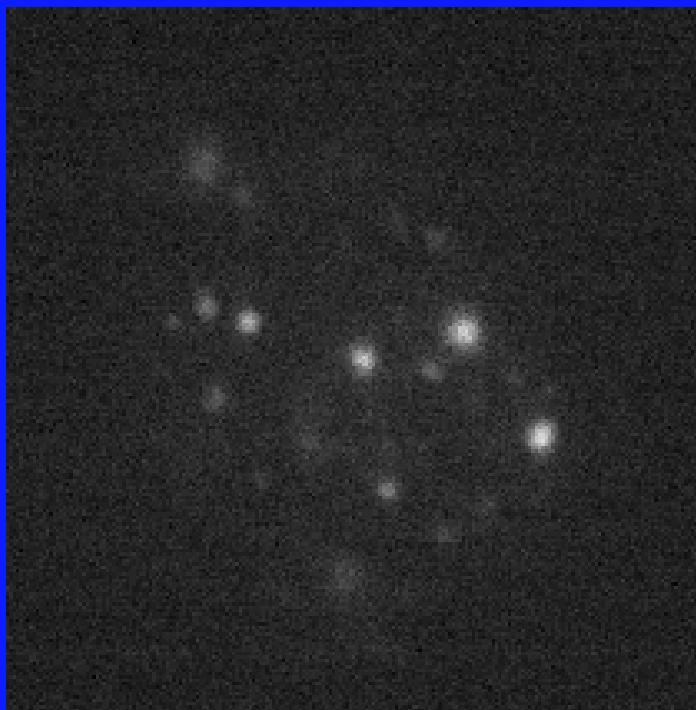
TIRF

QuickTime™ and a
YUV420 codec decompressor
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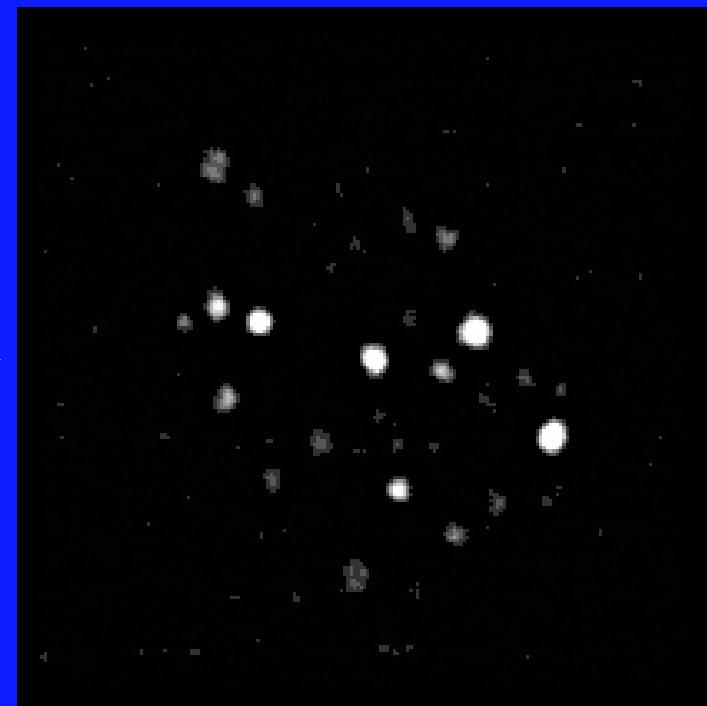
5Hz recording, 20Hz playback

Filtering images for better contrast

C



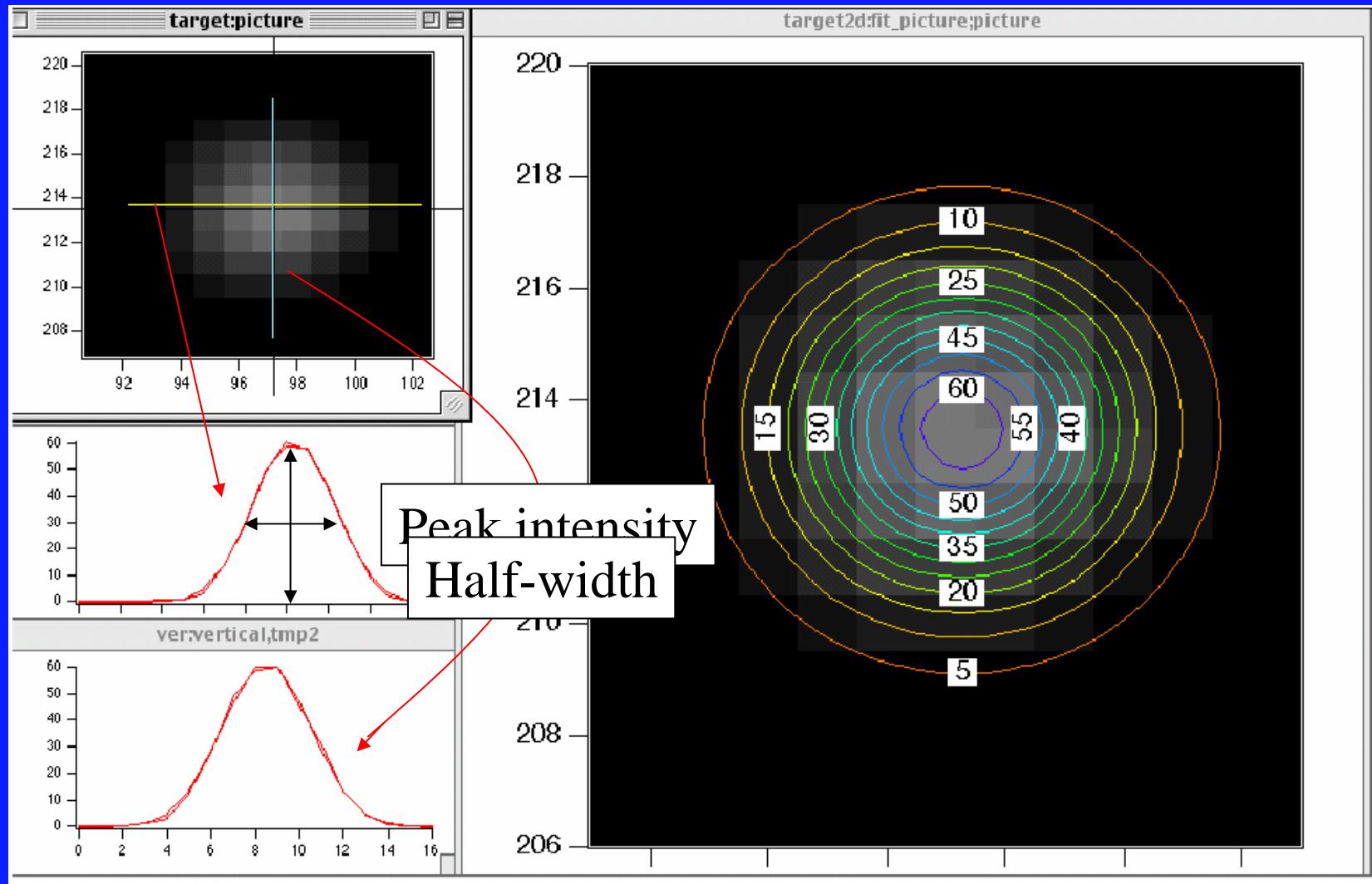
D



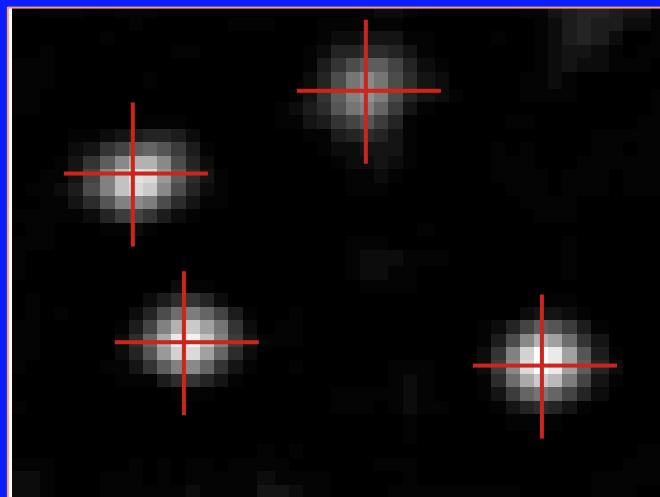
3 steps filtering



Identification of individual vesicles

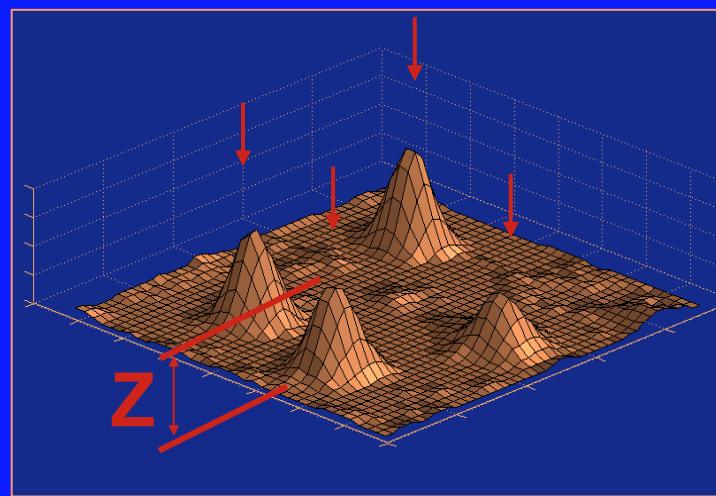


Identification of individual vesicles



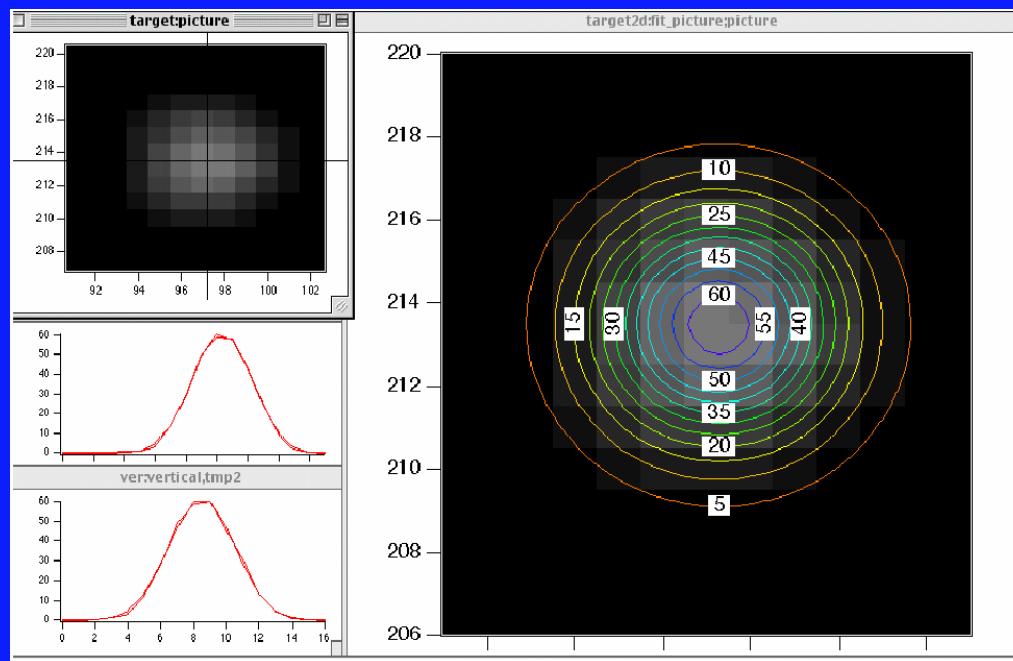
y

x

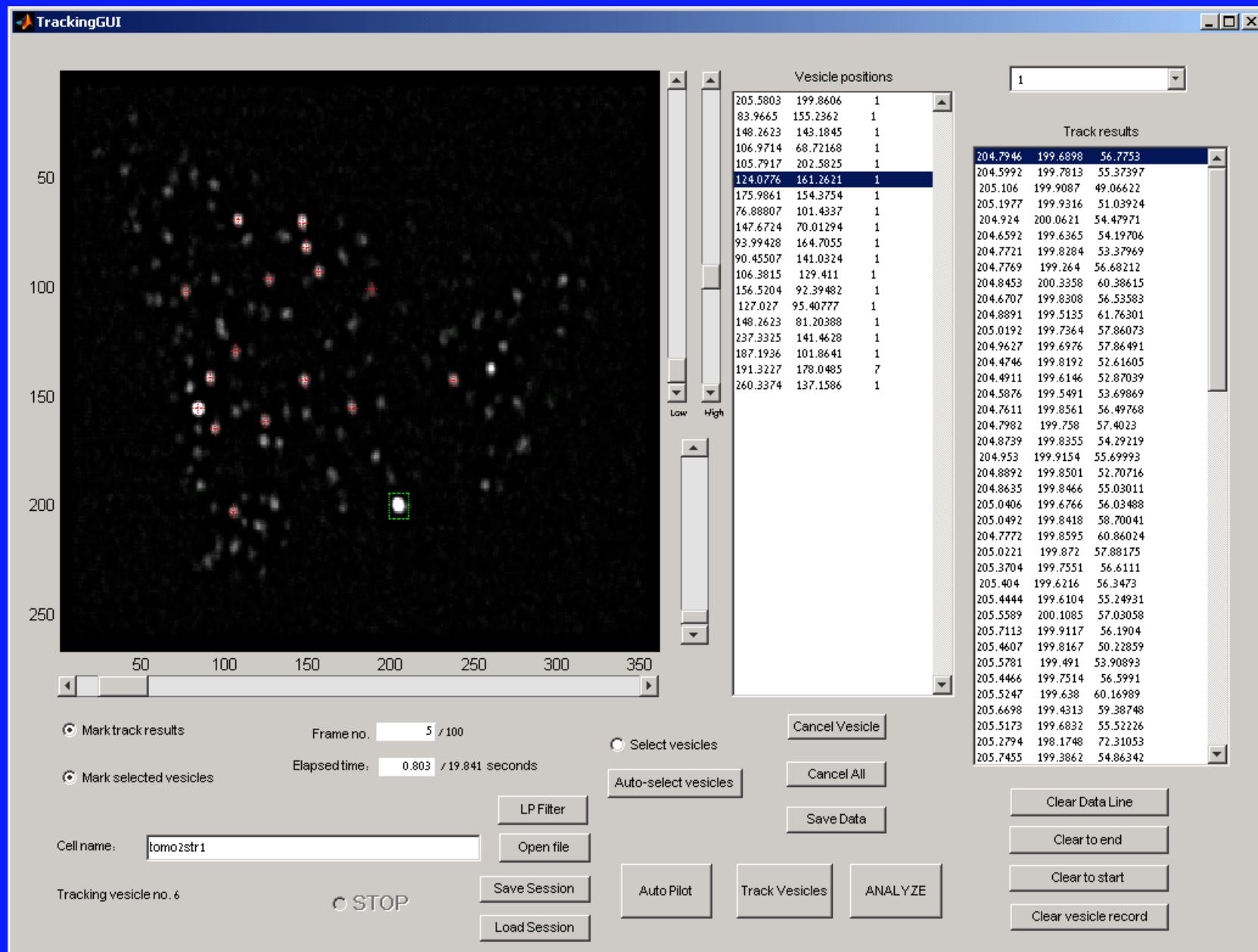


Information derived from image sequences:

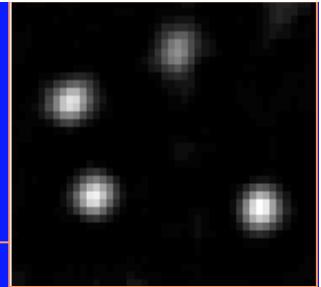
- X/Y position – By fitting the intensity distribution with a gaussian curve, finding the location of the peak
- Z position – indicated by peak intensity



Tracking single vesicles:



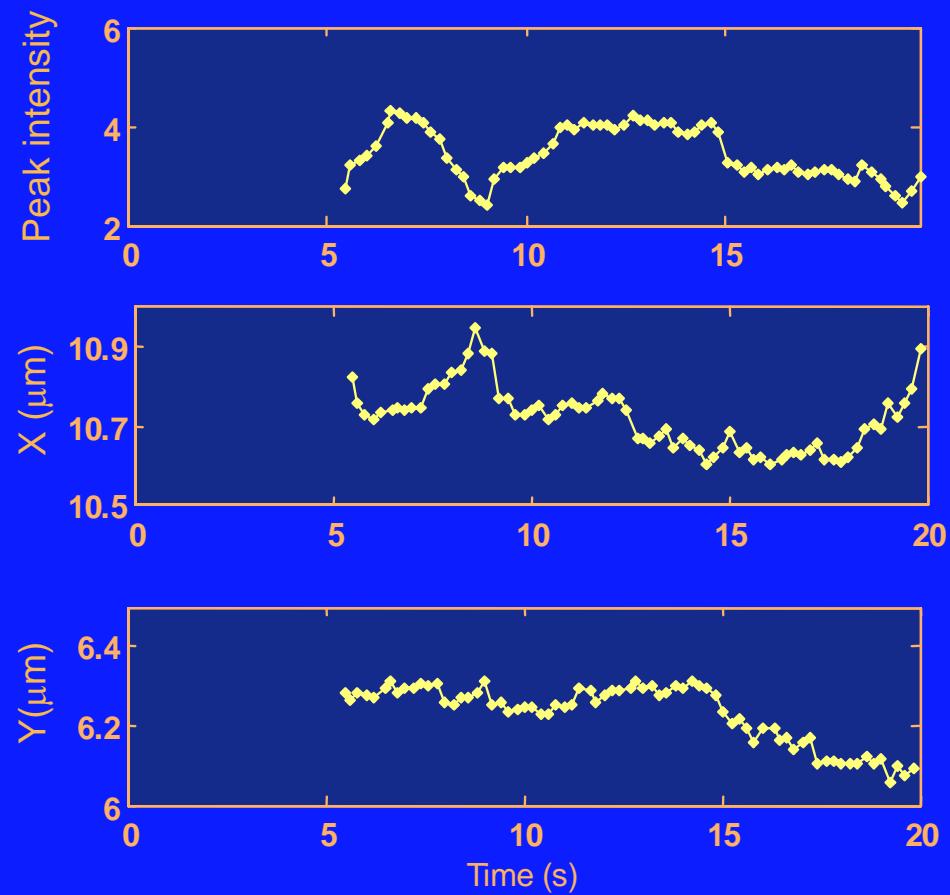
Tracking vesicle motion



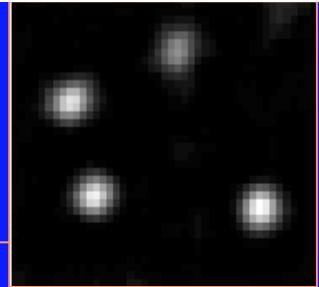
QuickTime™ and a
YUV420 codec decompressor
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5Hz recording, 20Hz playback

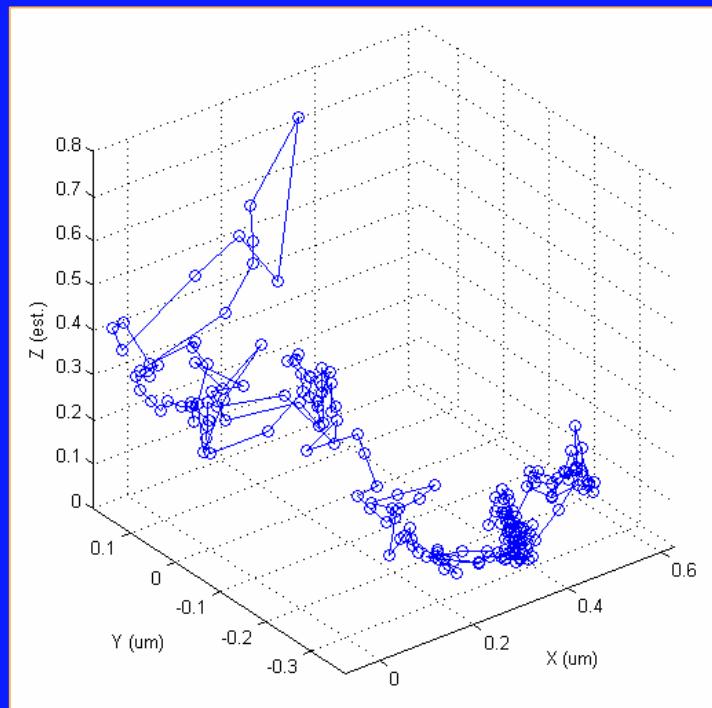
Data from tracking single vesicle



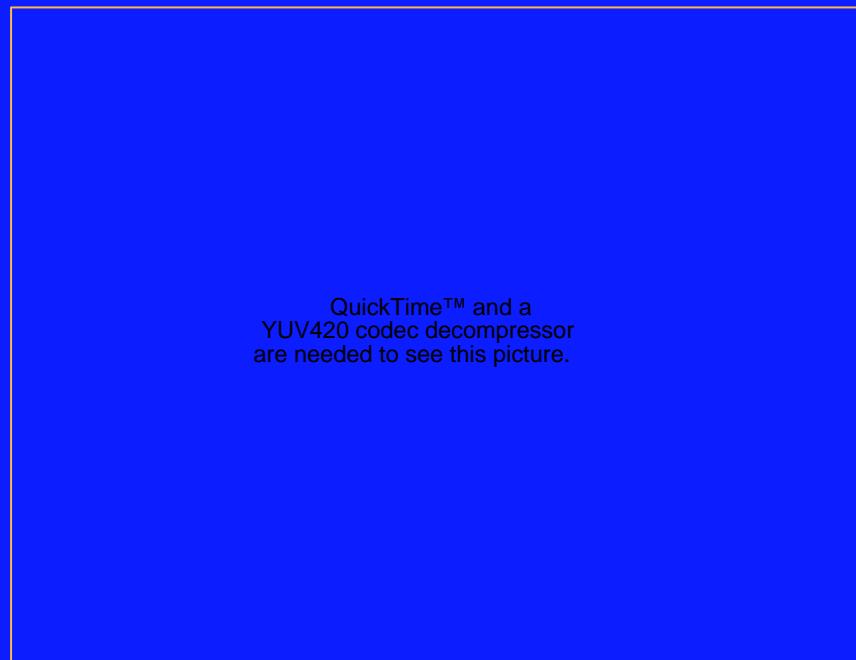
Tracking vesicle motion



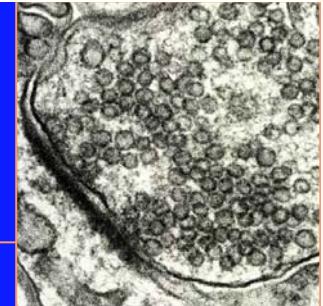
3-D trajectory of a single vesicle



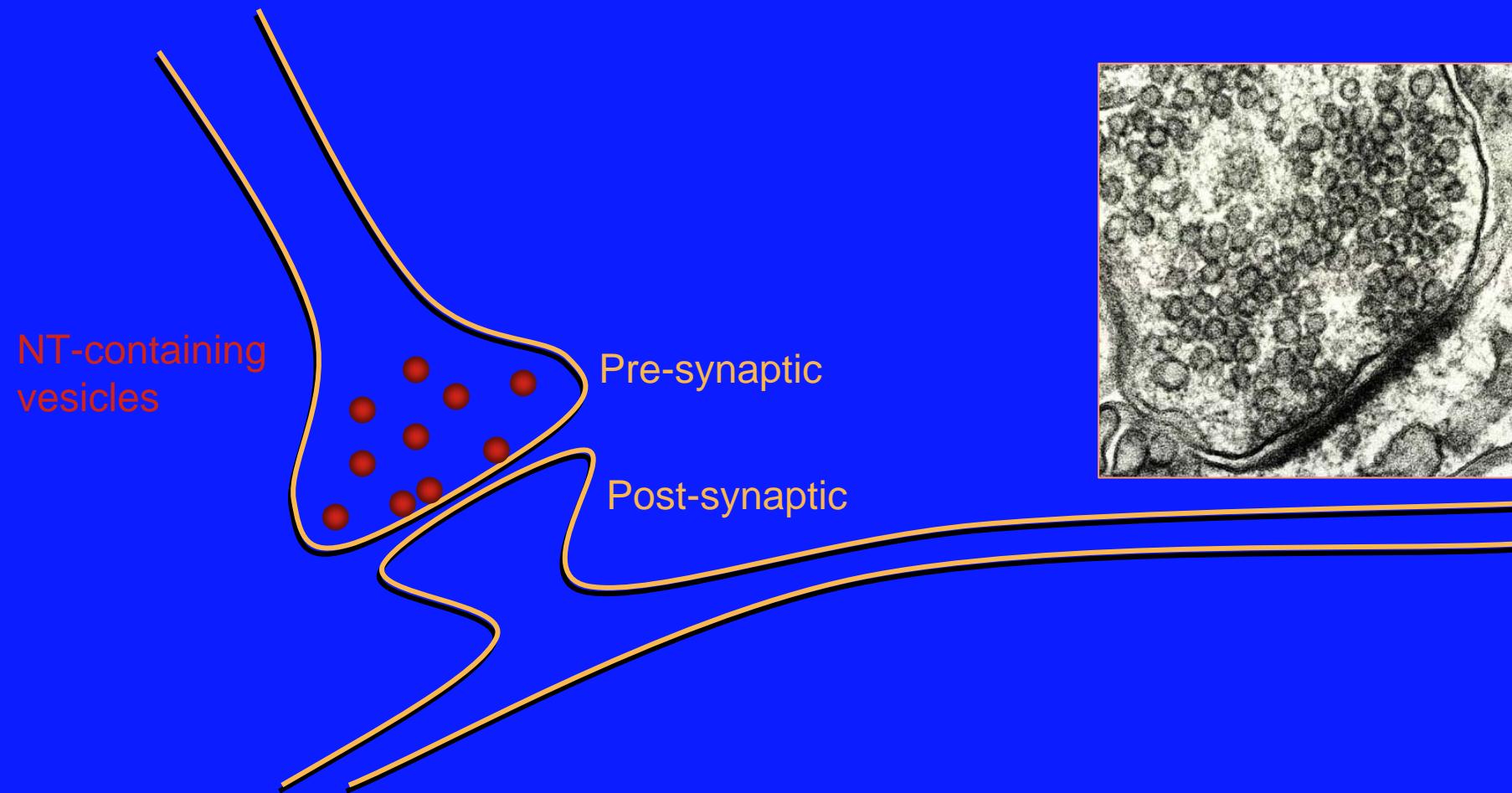
3-D reconstruction of vesicle trajectories



Synaptic transmission is carried out by fusion of neurotransmitter-containing vesicles

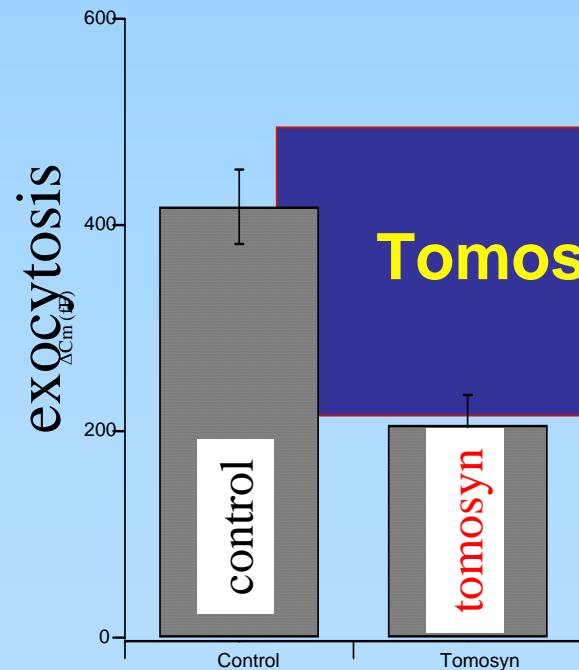


Molecular mechanisms of vesicle exocytosis and endocytosis:

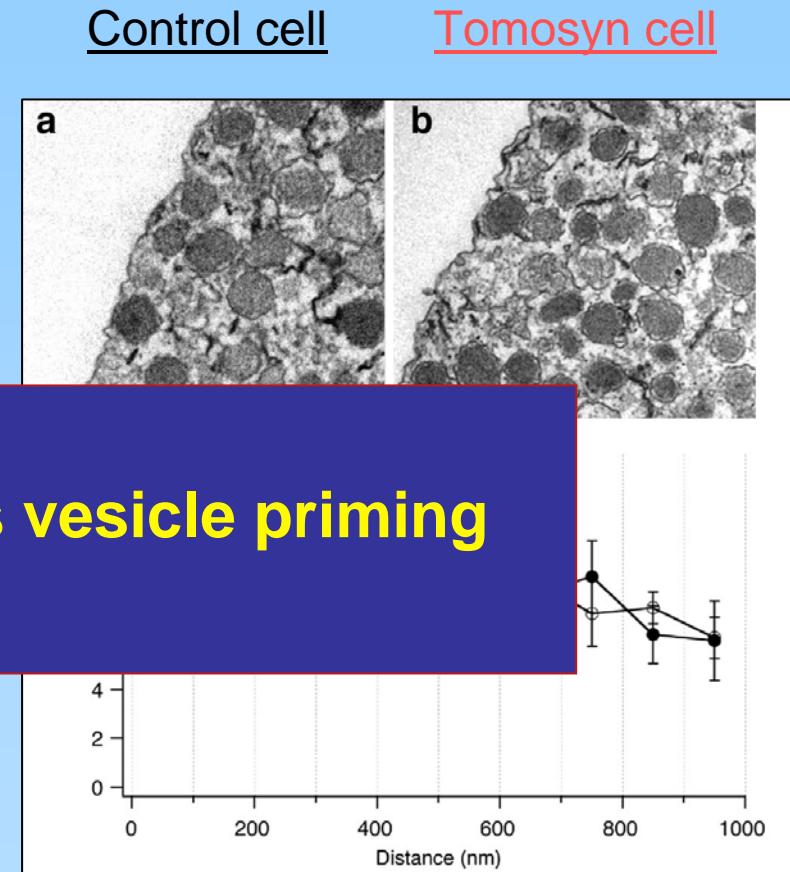


Tomosyn inhibits exocytosis

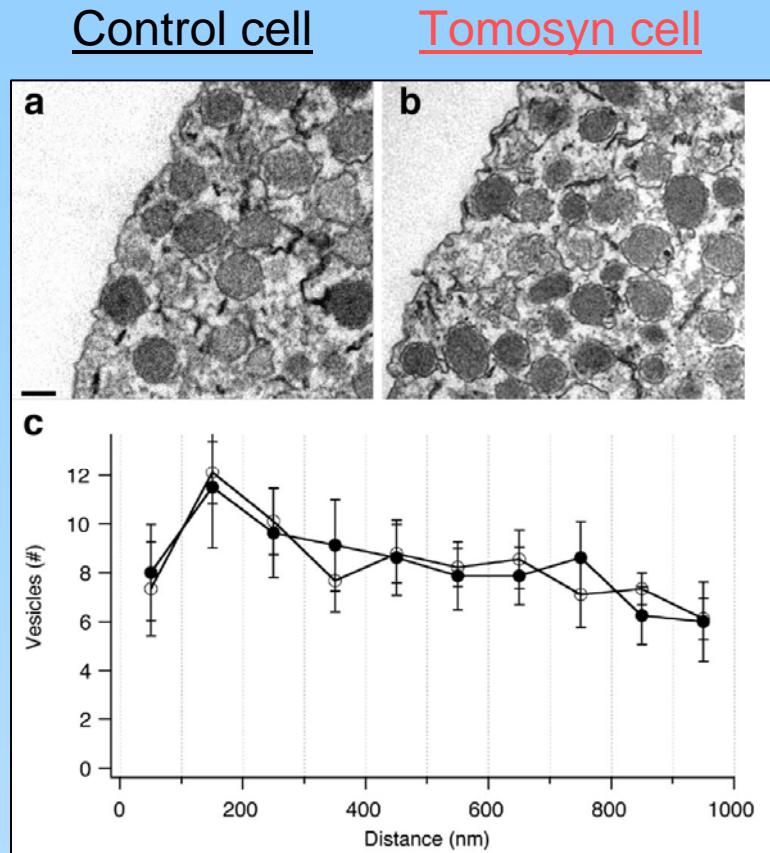
Tomosyn doesn't inhibit docking



Tomosyn inhibits vesicle priming



EM Analysis of Vesicle Distribution With Tomosyn Overexpression



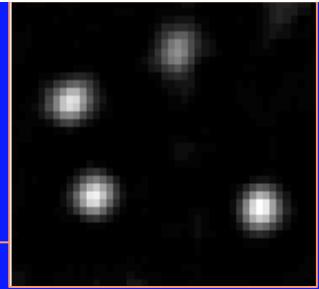
Docked

→ Primed

→ Fusion-Competent

Can we define physically primed vesicle?

Calculation of vesicle mobility

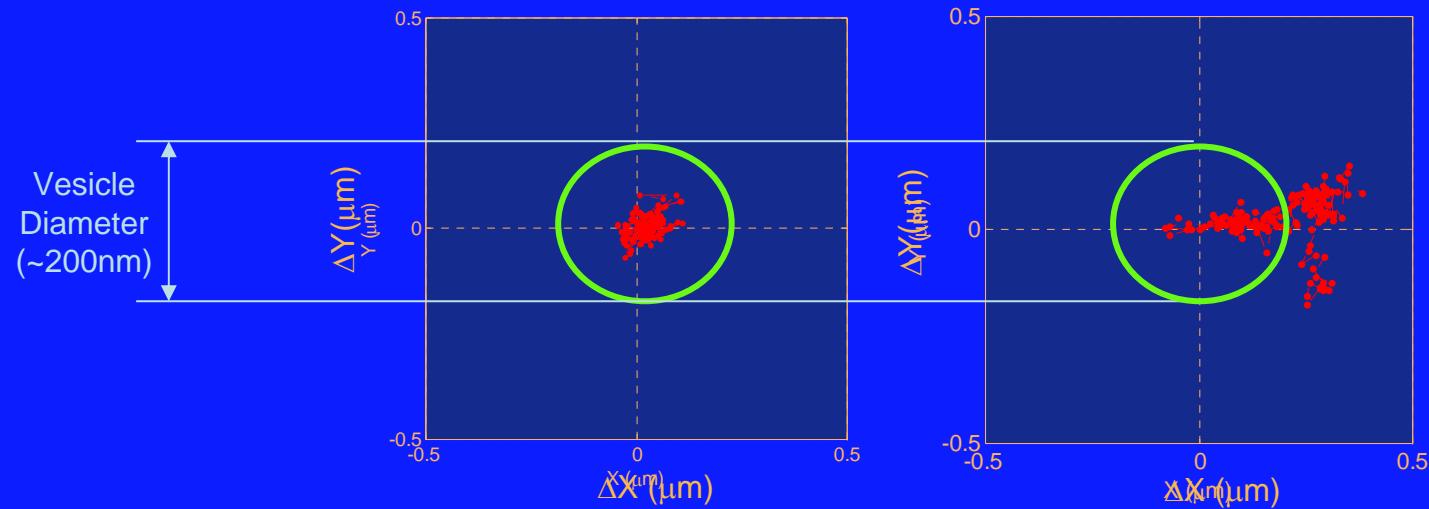


MSD plot

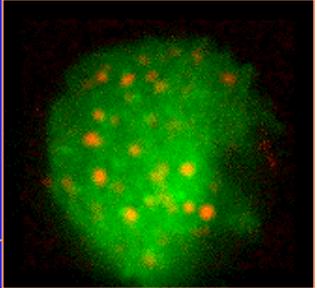


Diffusion constant

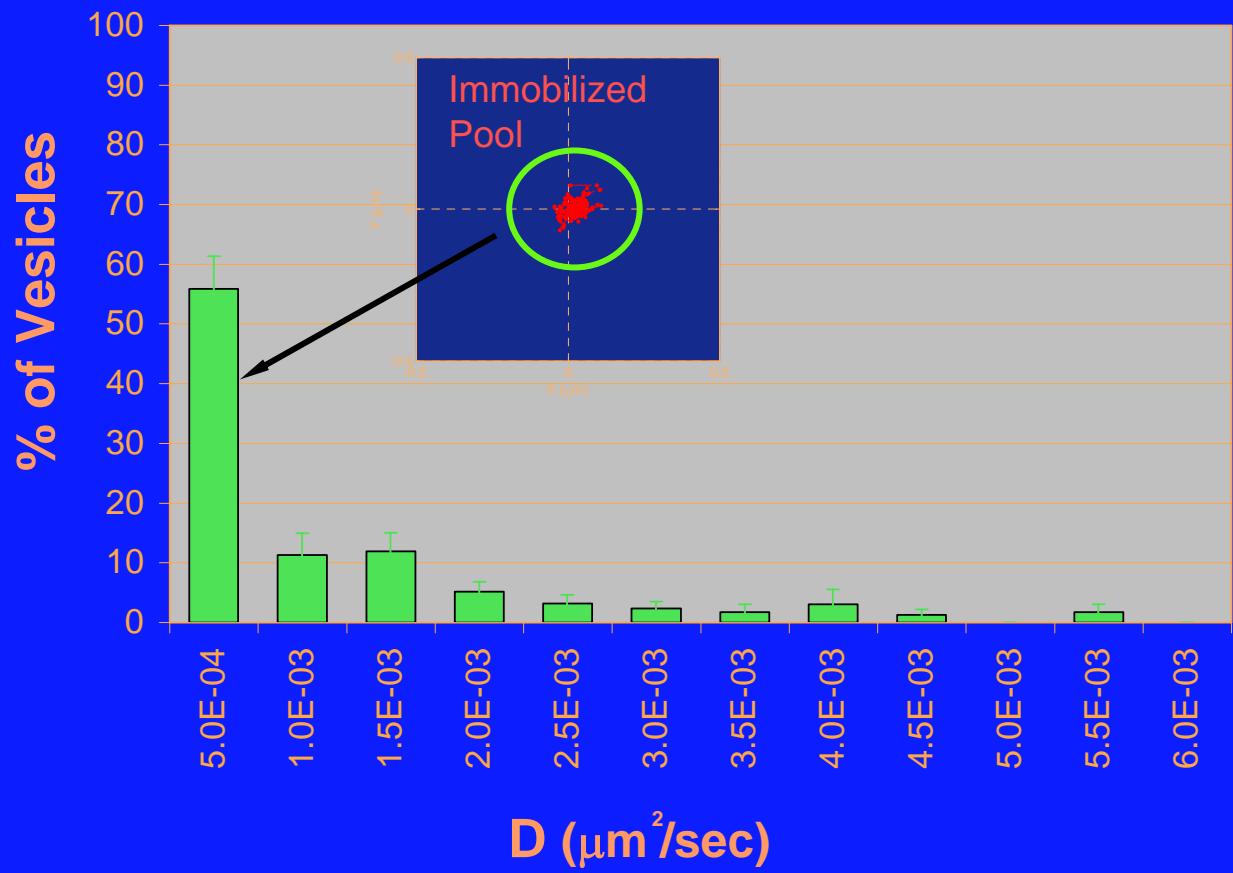
$$D = 1.65 \times 10^{-4} (\mu\text{m}^2/\text{sec}) \quad D = 1.39 \times 10^{-3} (\mu\text{m}^2/\text{sec})$$



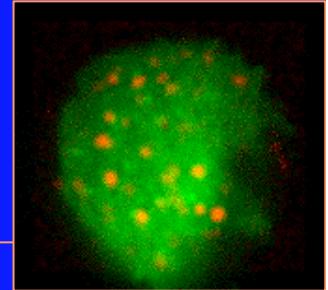
In untreated cells, ~50% of membrane-proximal vesicles are immobilized



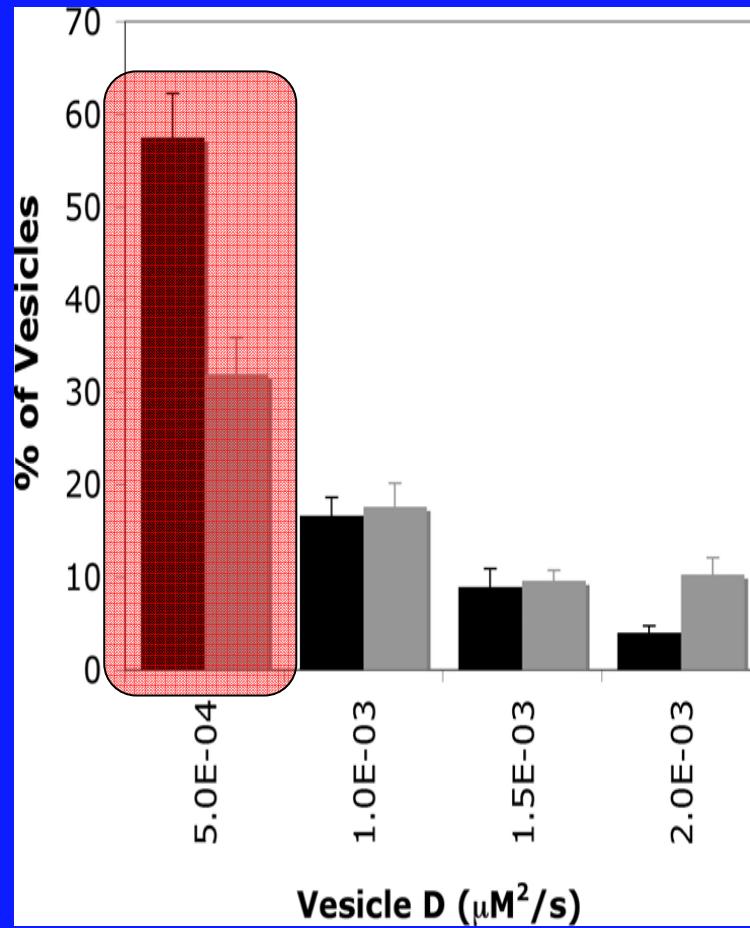
Distribution of vesicle diffusion coefficients in untreated cells:



Studying the roles of synaptic proteins through vesicle mobility



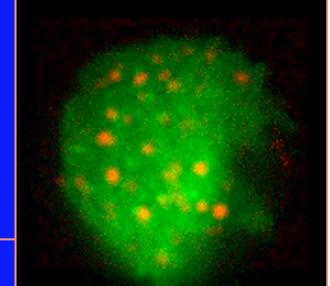
Tomosyn reduces the population of immobilized vesicles



Is this immobile pool represent ready-to-fuse vesicles?

Yizhar et al.

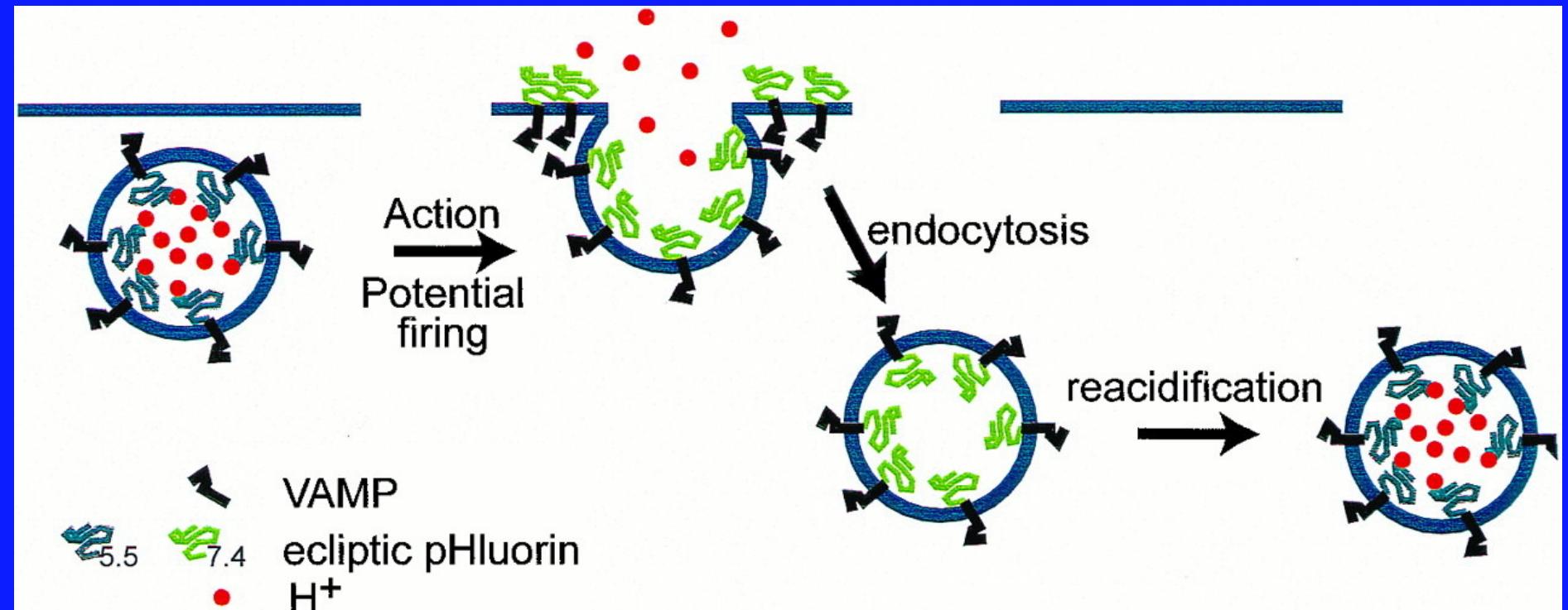
Studying the roles of synaptic proteins through vesicle mobility; vesicle fusion



QuickTime™ and a
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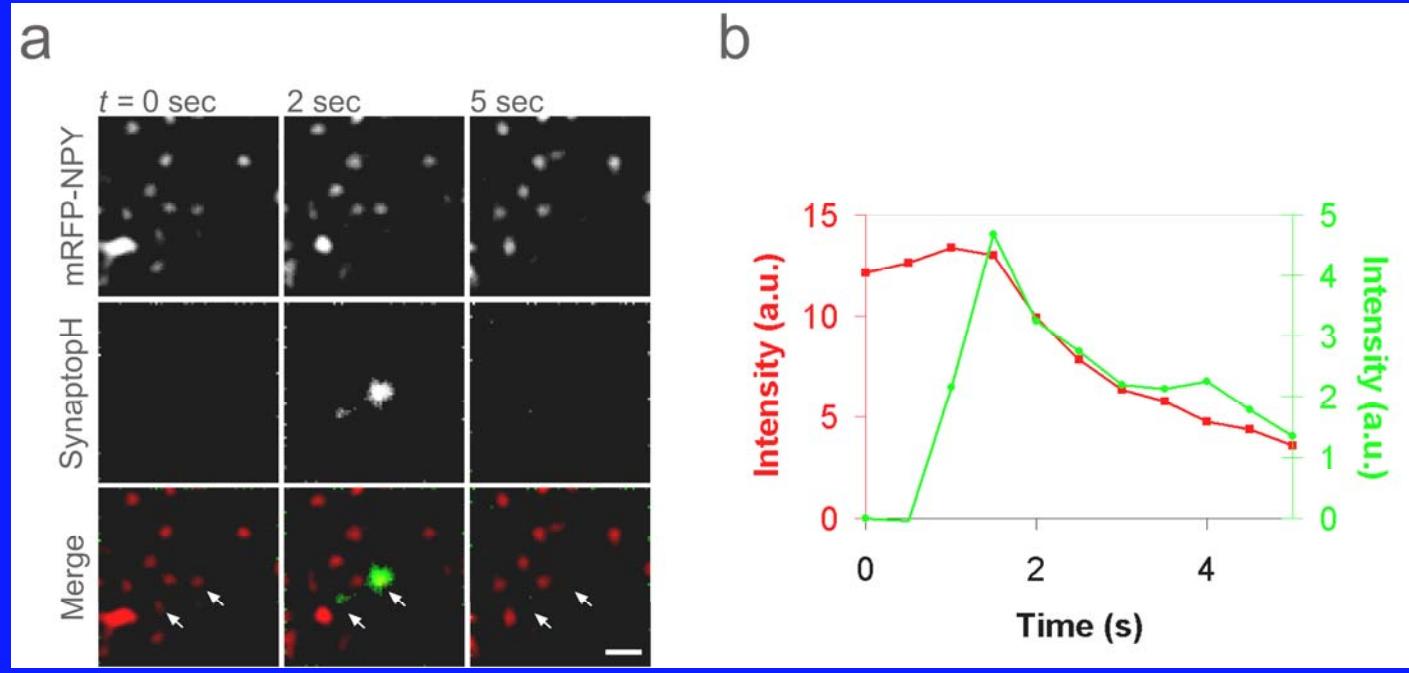
Do we measure fusion or undocking?

pH-sensitive green fluorescent protein (pHluorin) fused to Synatpobrevin (SynaptopHluorin)

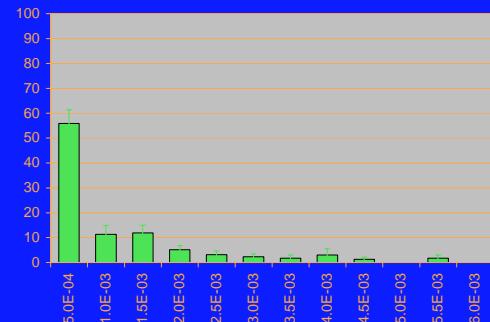
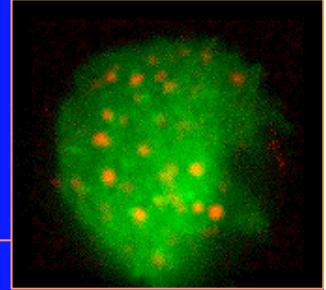


Exocytosis relieves the proton-dependent quenching of ecliptic-pHluorin fluorescence

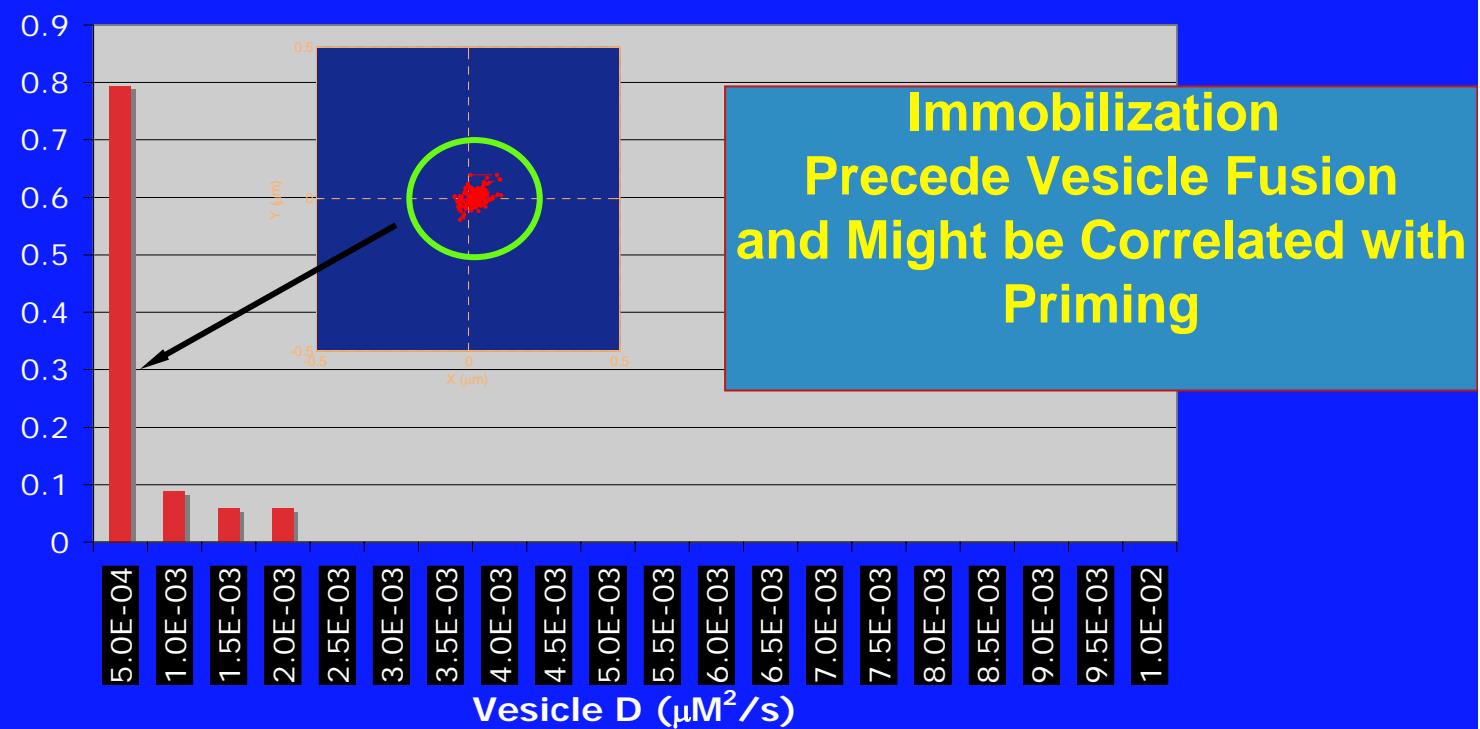
Dual color TIRF imaging of NPY-mRFP and Synaptophysin



Diffusion constants of vesicles immediately before fusion

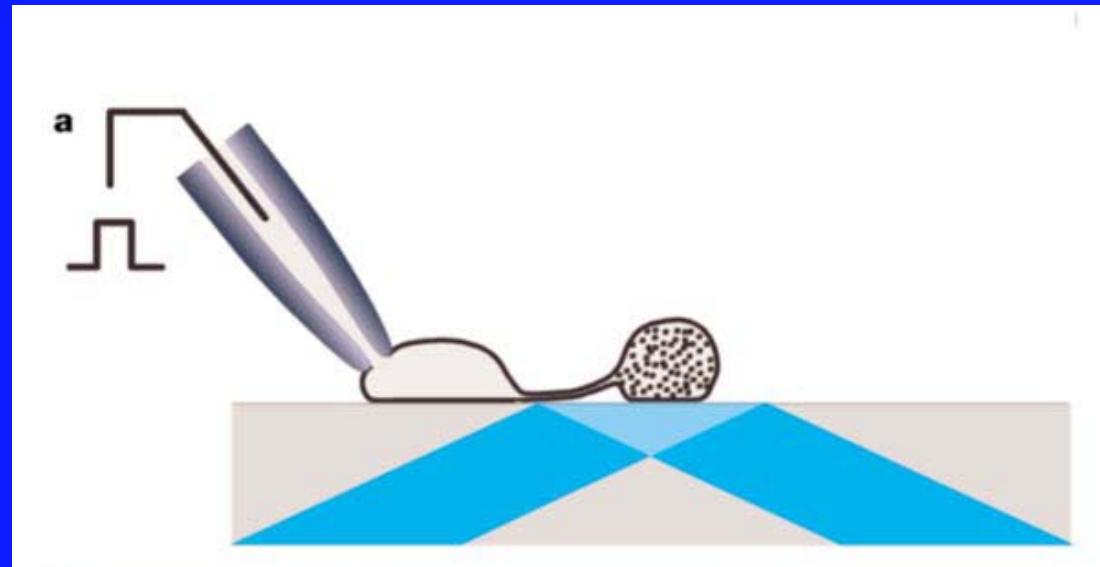


80% of pre-fusion vesicles are immobilized



Yizhar et al.

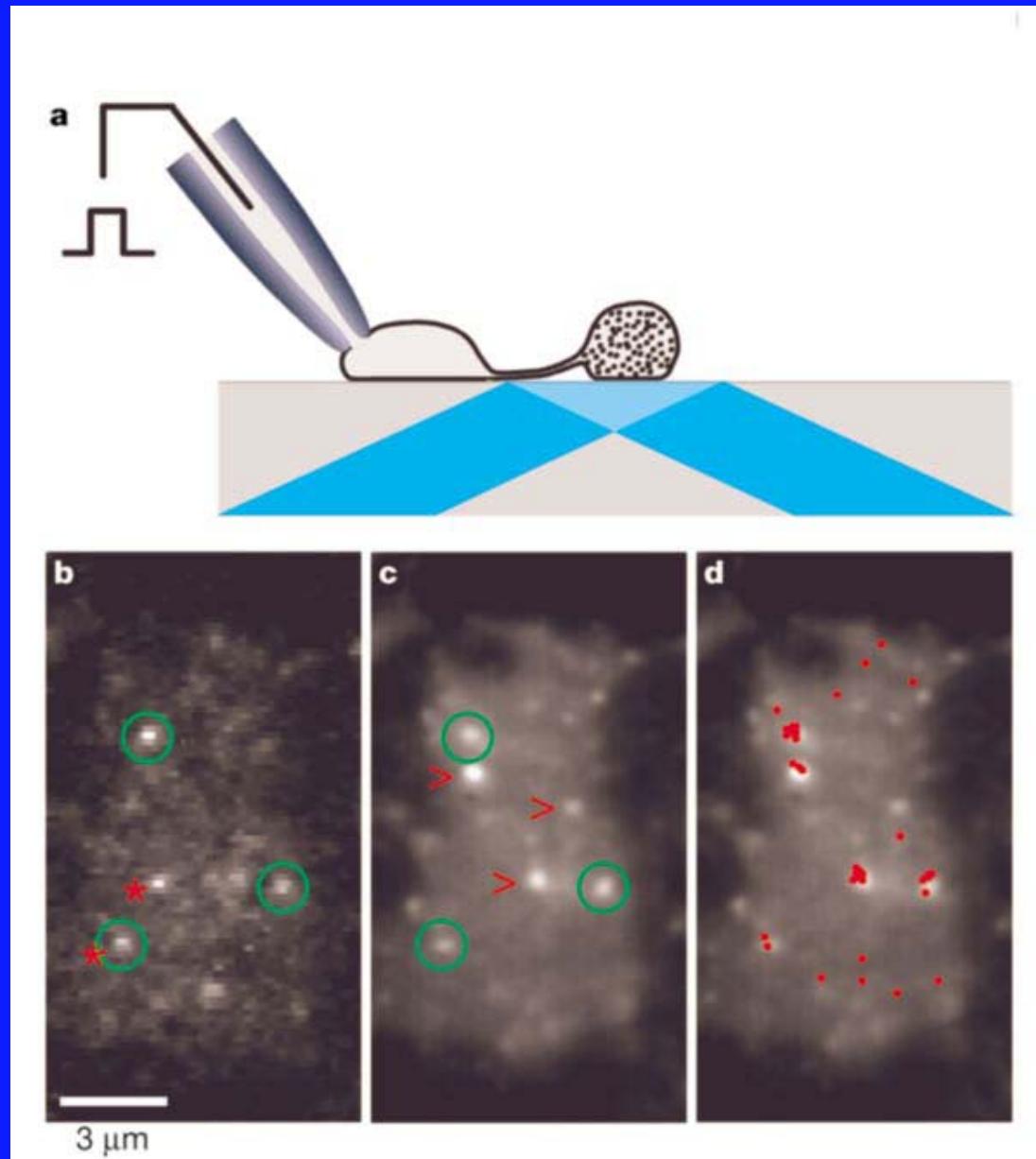
Exocytosis of single synaptic vesicles (diameter 50nm) in Retinal bipolar neurons



QuickTime™ and a
Animation decompressor
are needed to see this picture.

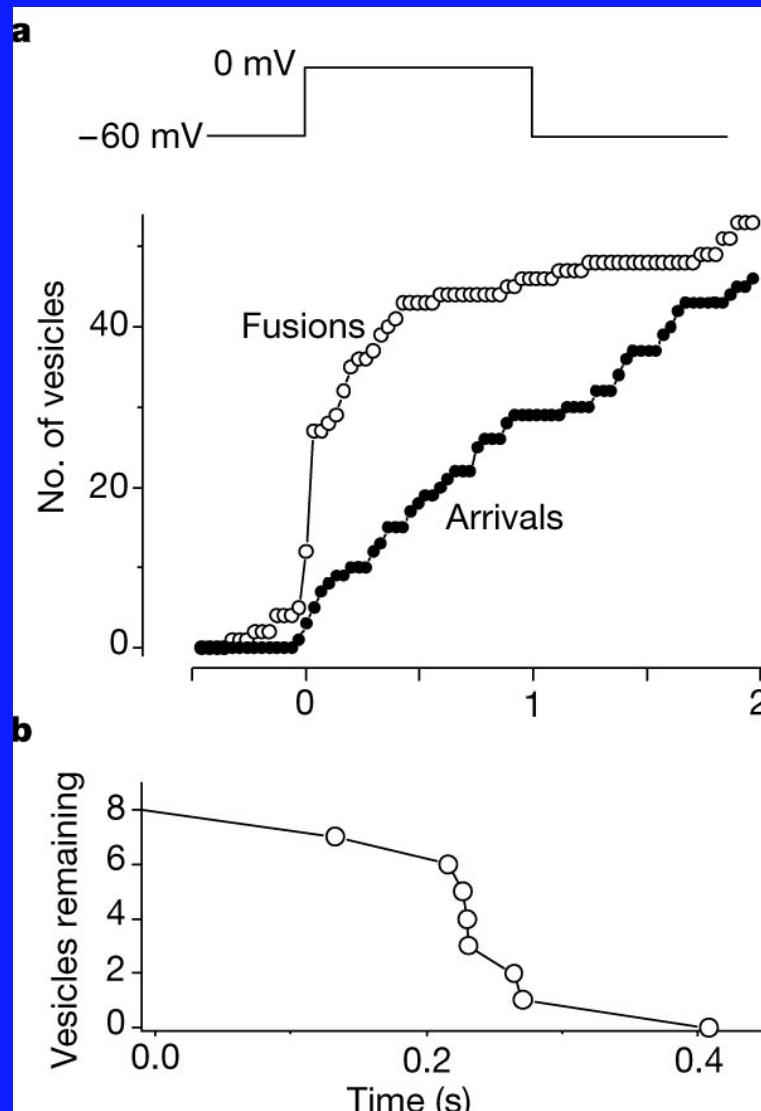
QuickTime™ and a
Animation decompressor
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Hot spots for exocytosis in Retinal bipolar neurons



Zenisek, Steyer, Almers 2000

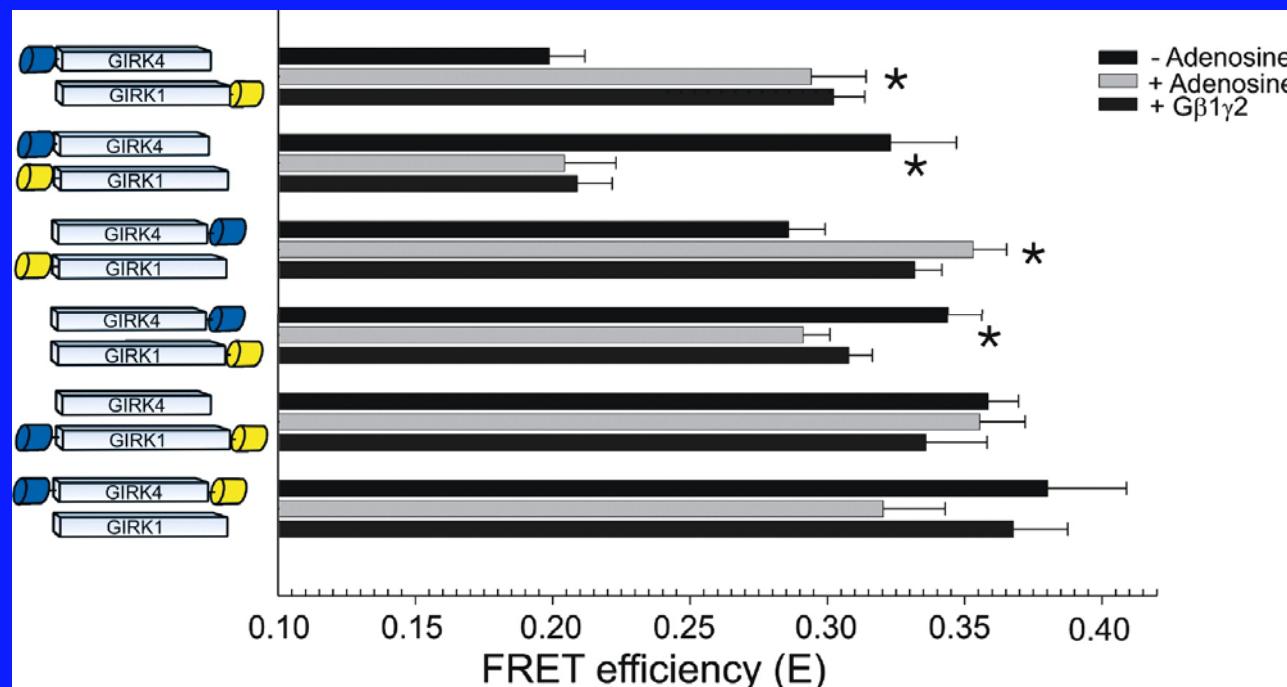
Exocytosis of different pools of vesicles in Retinal bipolar neurons



Zenisek, Steyer, Almers 2000

Conformational rearrangements associated with the gating of G protein-coupled Potassium channel

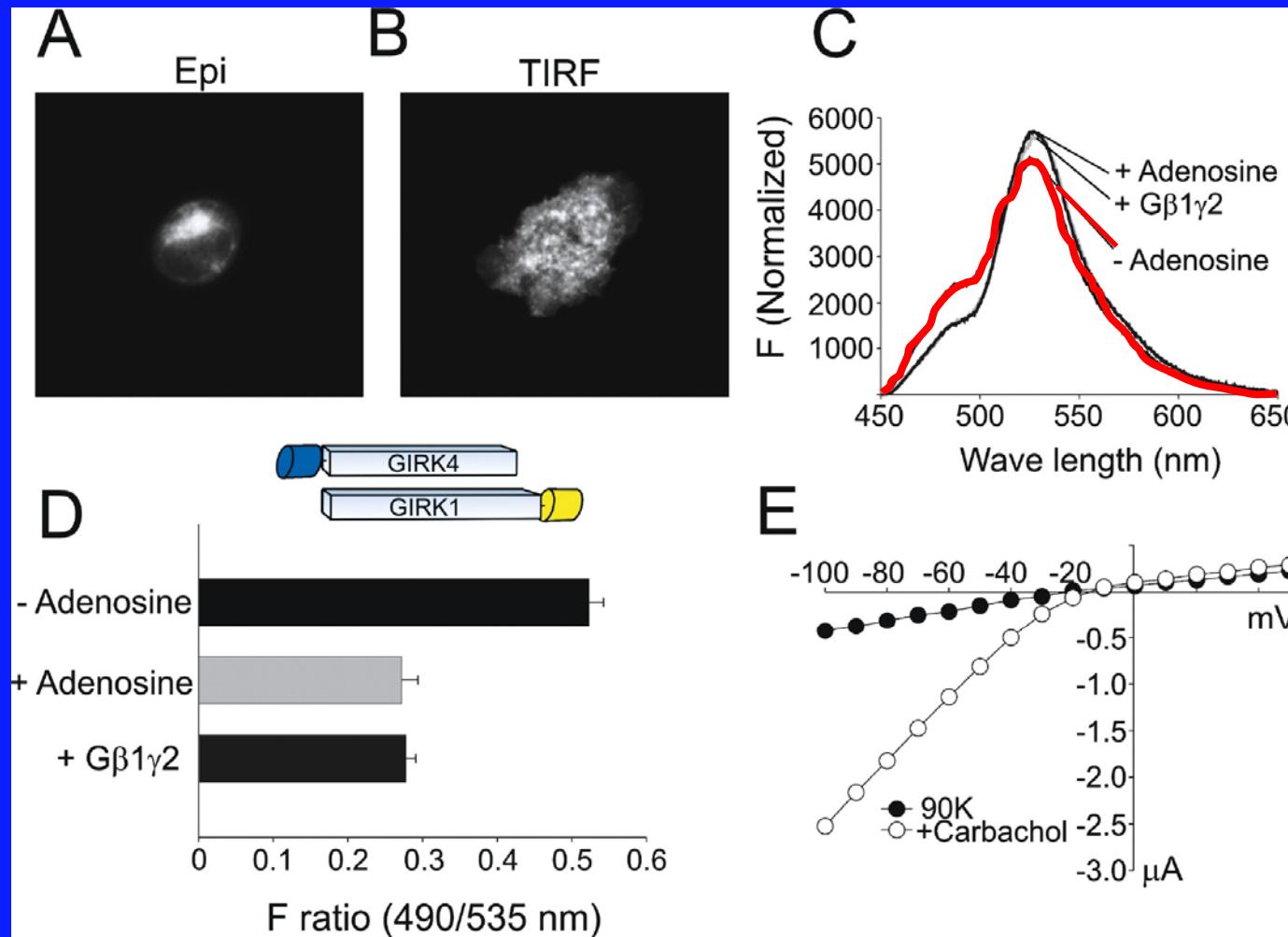
Spectroscopic Measurements of FRET under TIRF Microscopy



Inbal Riven , Eli Kalmanzon , Lior Segev , and Eitan Reuveny
Neuron, Vol 38, 225-235, 24 April 2003

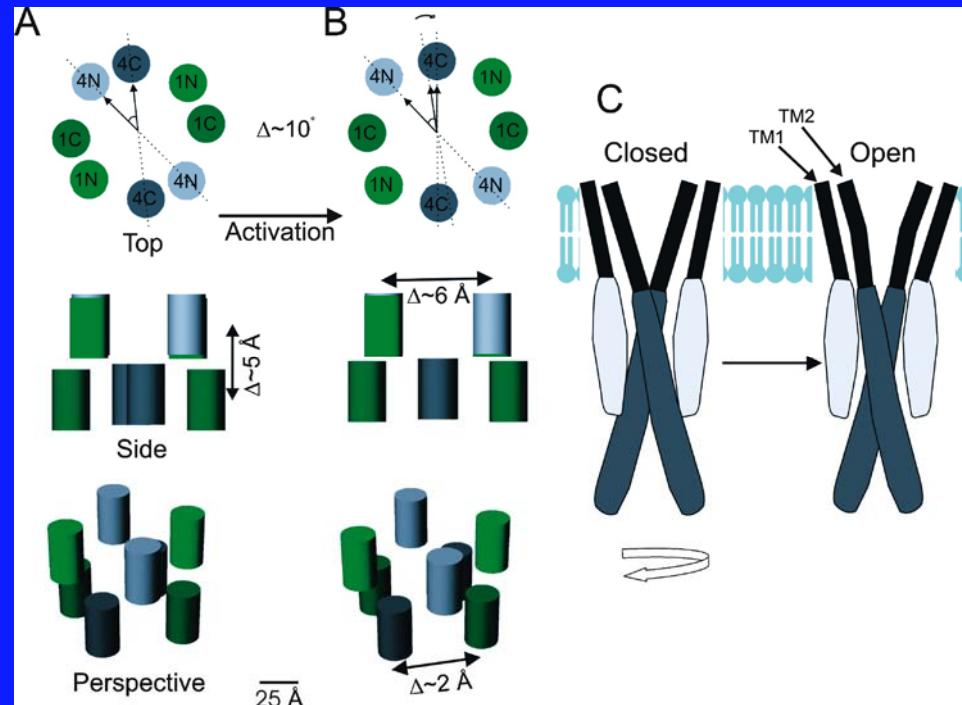
Spectroscopic Measurements of FRET under TIRF Microscopy

FRET Efficiencies Change during Channel Gating



Inbal Riven , Eli Kalmanzon , Lior Segev , and Eitan Reuveny
Neuron, Vol 38, 225-235, 24 April 2003

A Model Representing the Possible Rearrangement of the N- and C-Terminal Cytosolic Domains during Channel Gating

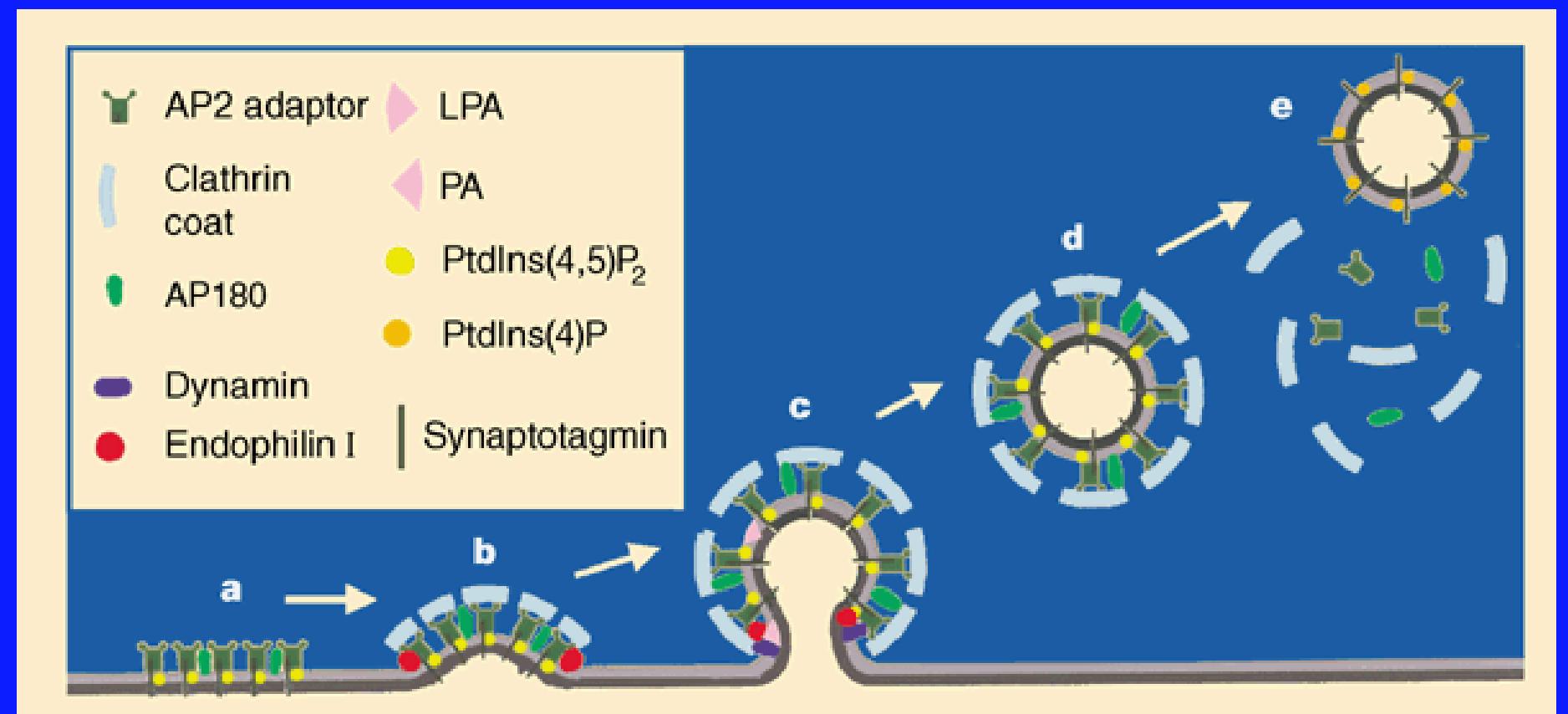


Since FRET efficiency depends heavily on the distance between the donor and the acceptor (R^{-6} power dependence) one can estimate distances between subunits.

Clathrin mediated endocytosis

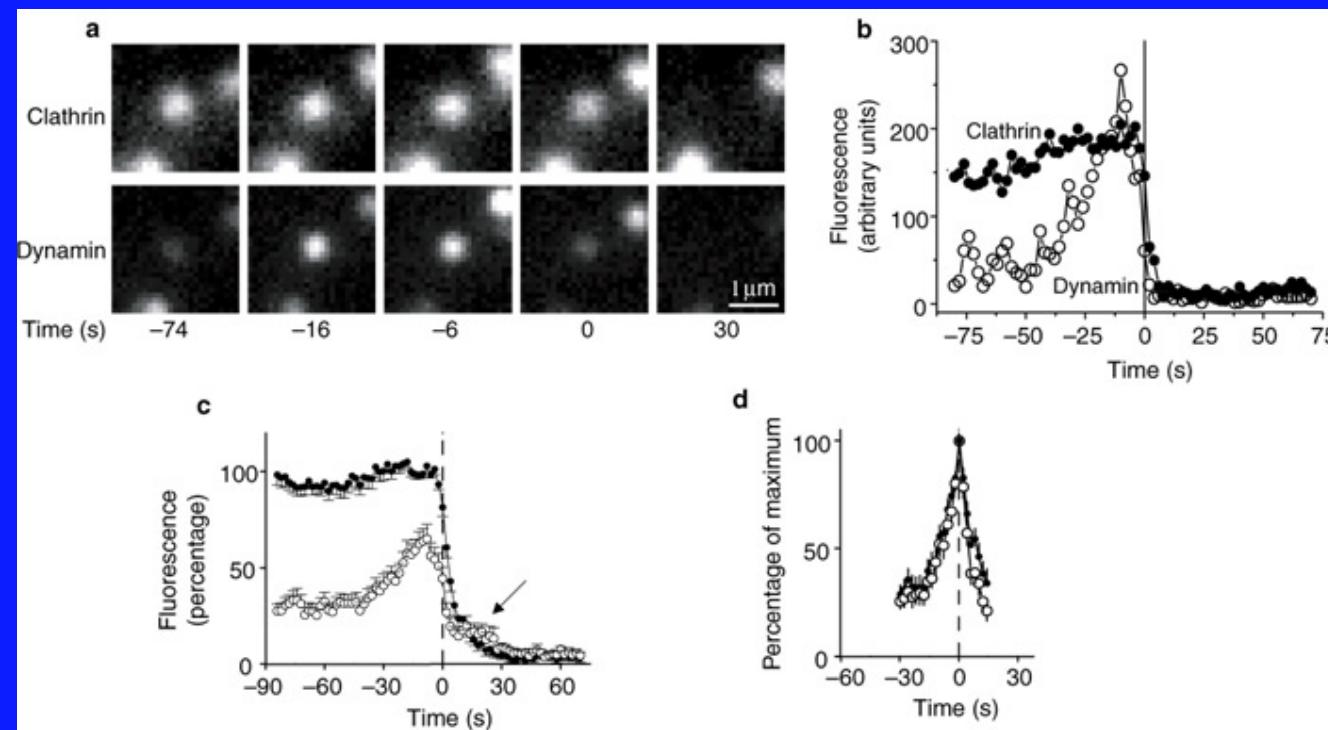
Clathrin: coat assembly

Dynamin: GTPase activity is needed for pinching off

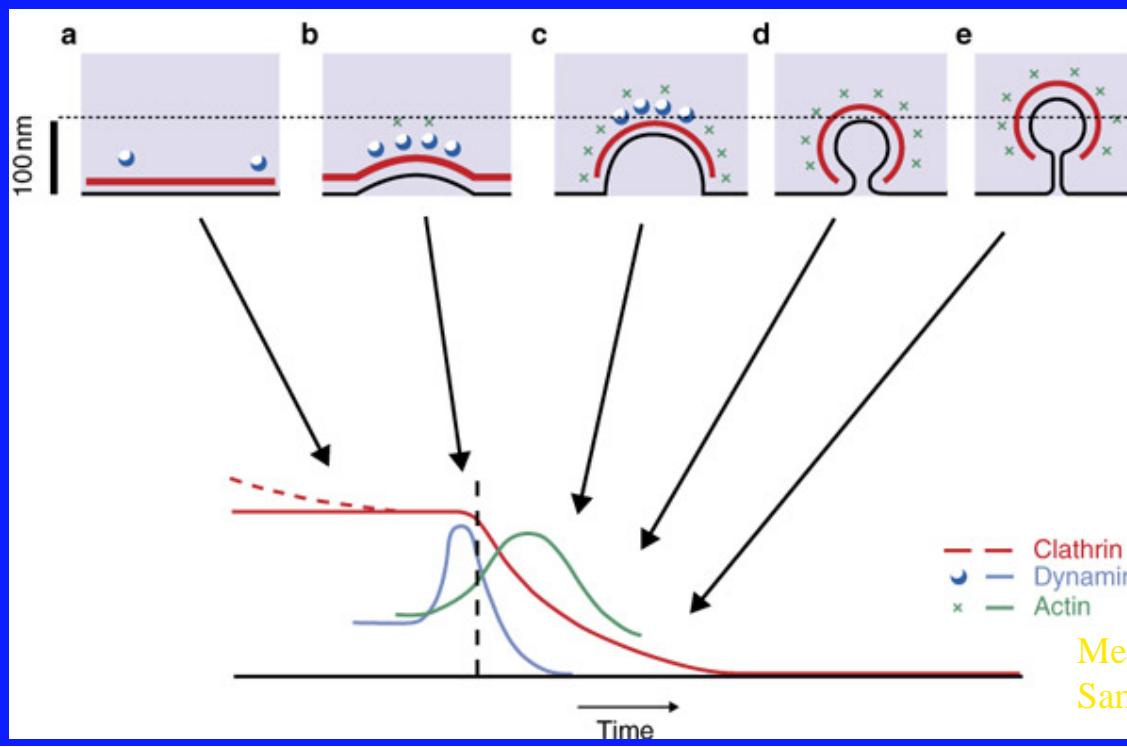
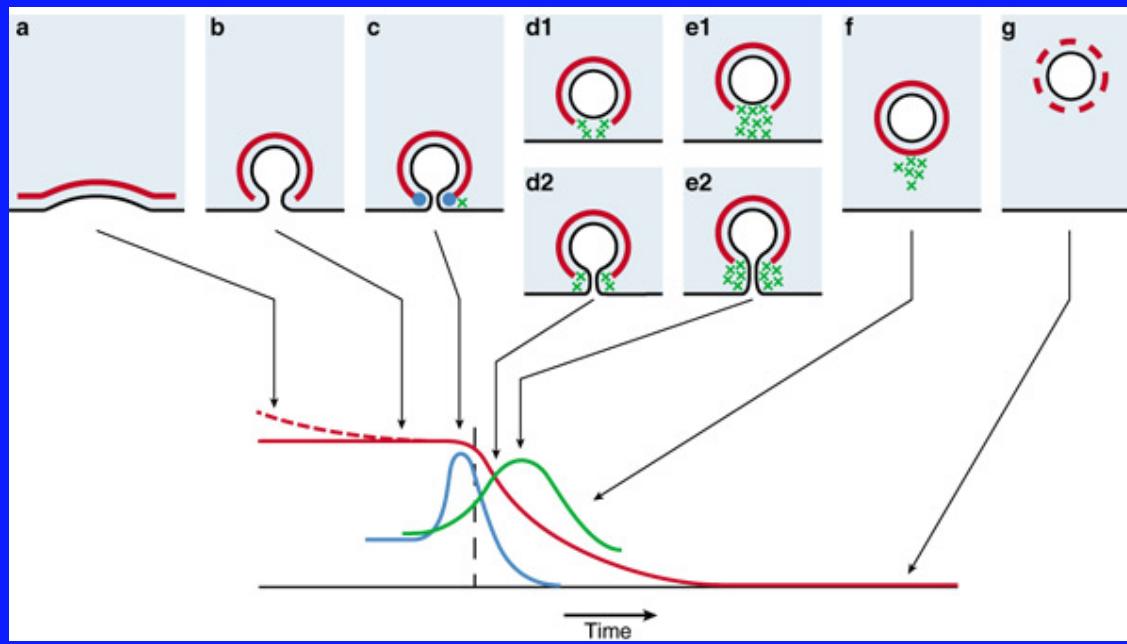


Slepnev and De Camilli 2000

Tracking endocytosis with TIRF

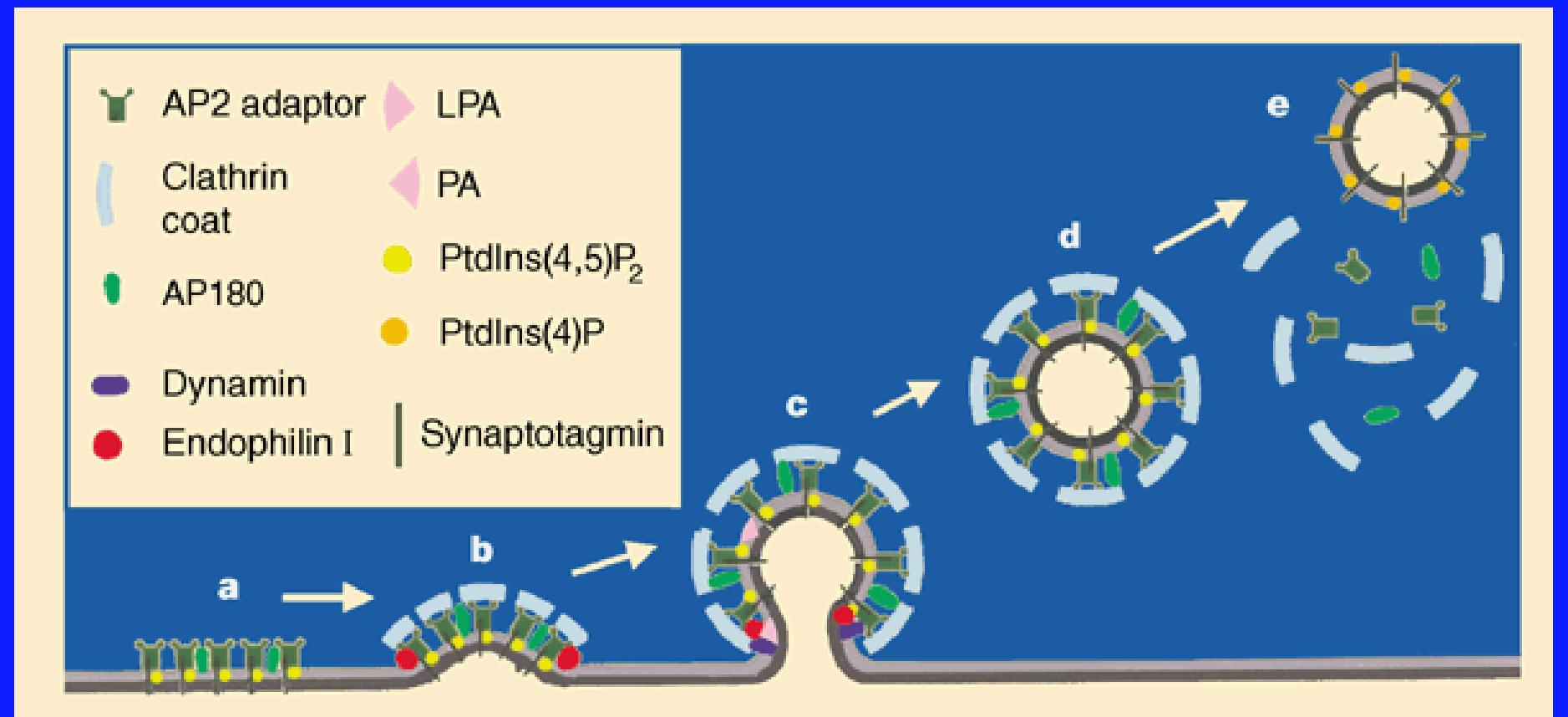


Merrifield et al, Almers 2002
Santini and Keen 2002



Merrifield et al, Almers 2002
Santini and Keen 2002

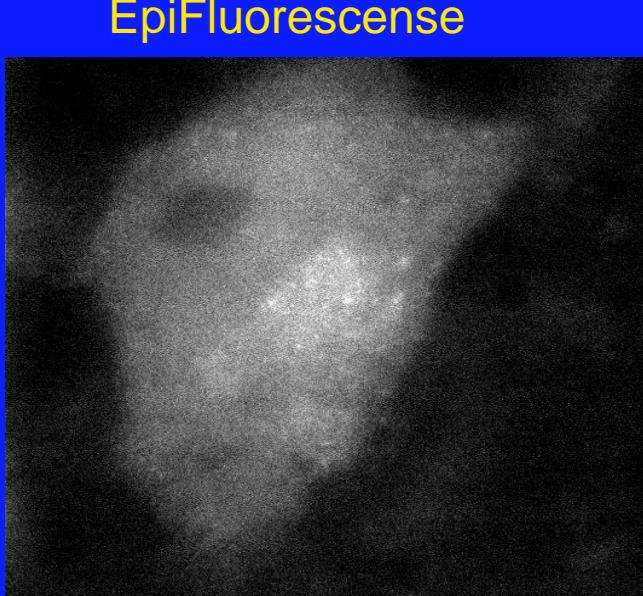
The role of Huntington interacting protein (HIP1) in clathrin mediated endocytosis



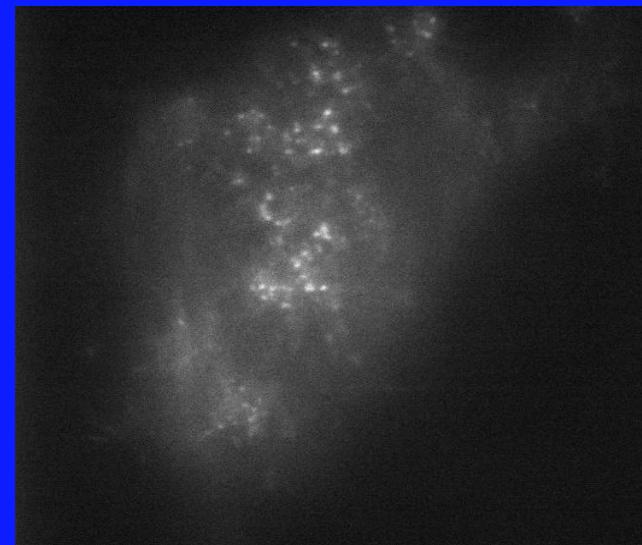
Slepnev and De Camilli 2000

The role of HIP1 in clathrin mediated endocytosis

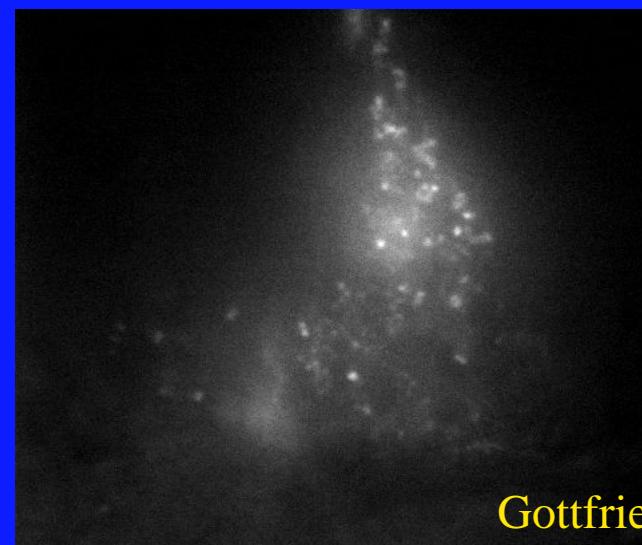
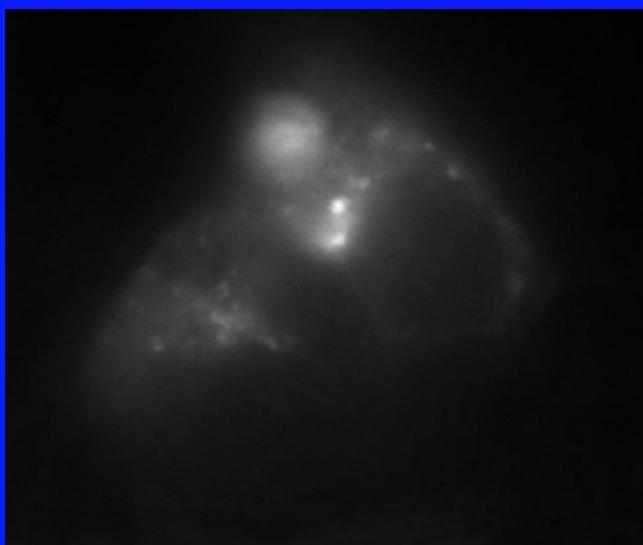
Clathrin-EGFP



TIRFM

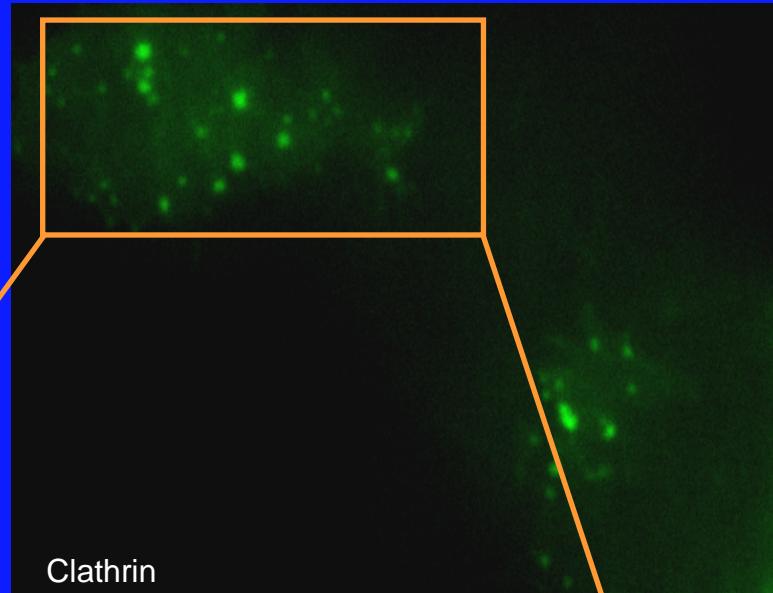
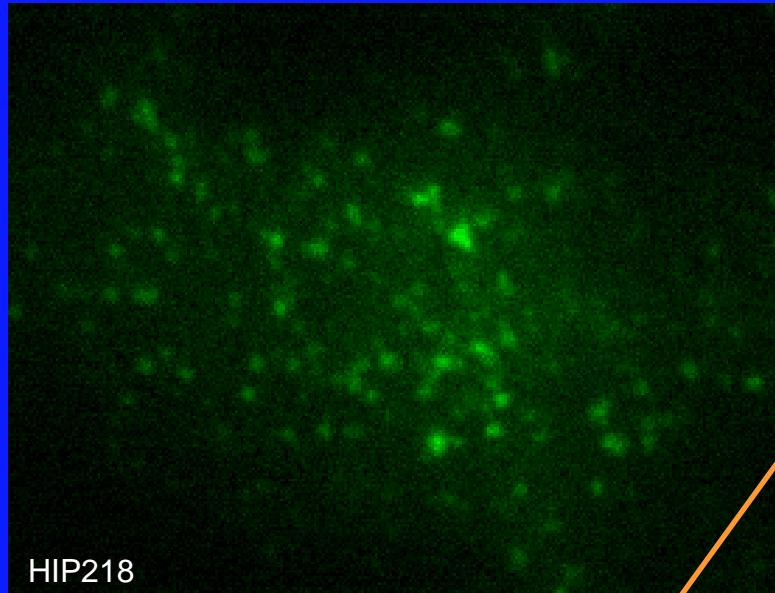


HIP218-EGFP



Gottfried et al.

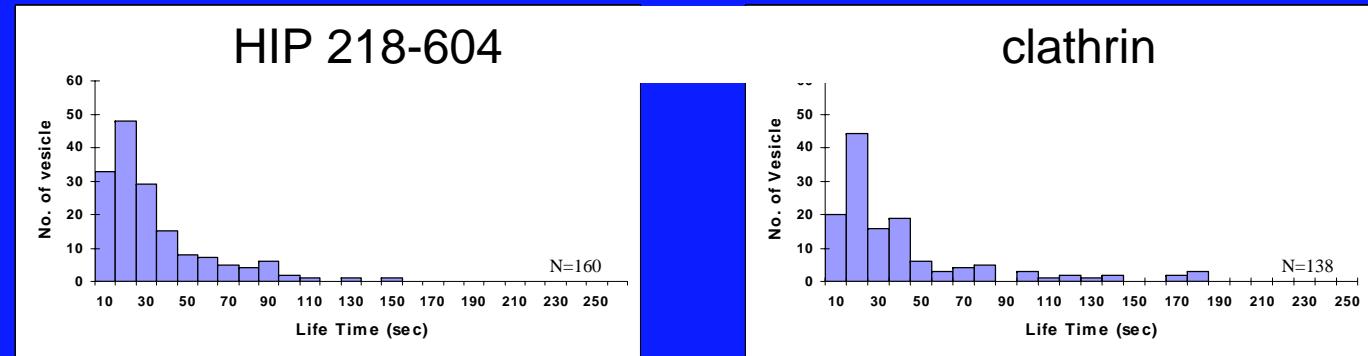
Behavior of HIP1 and clathrin in live cos7 cells



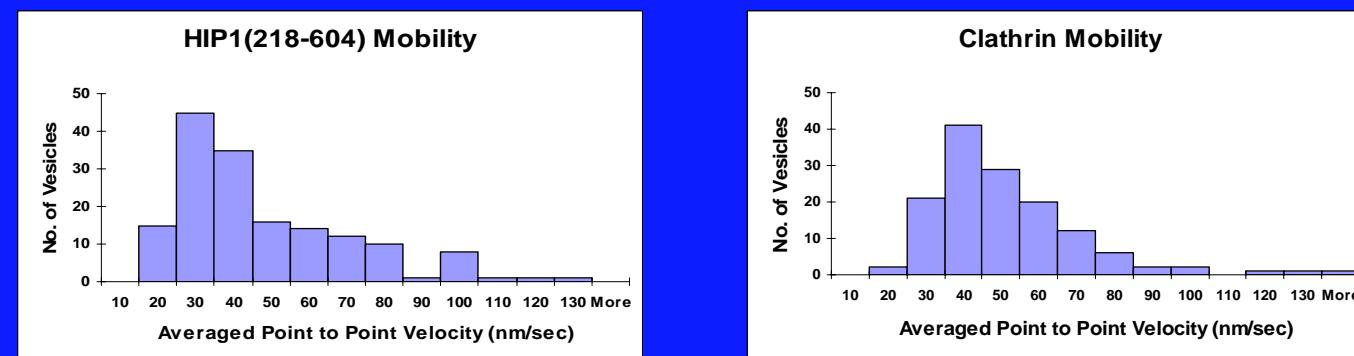
QuickTime™ and a
YUV420 codec decompressor
are needed to see this picture.

Kinetic analysis of HIP and clathrin clusters

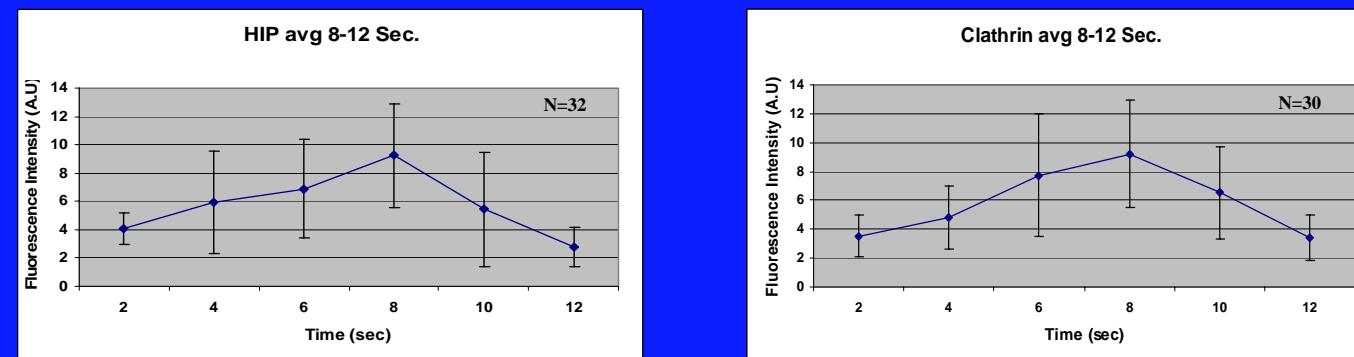
Life Time



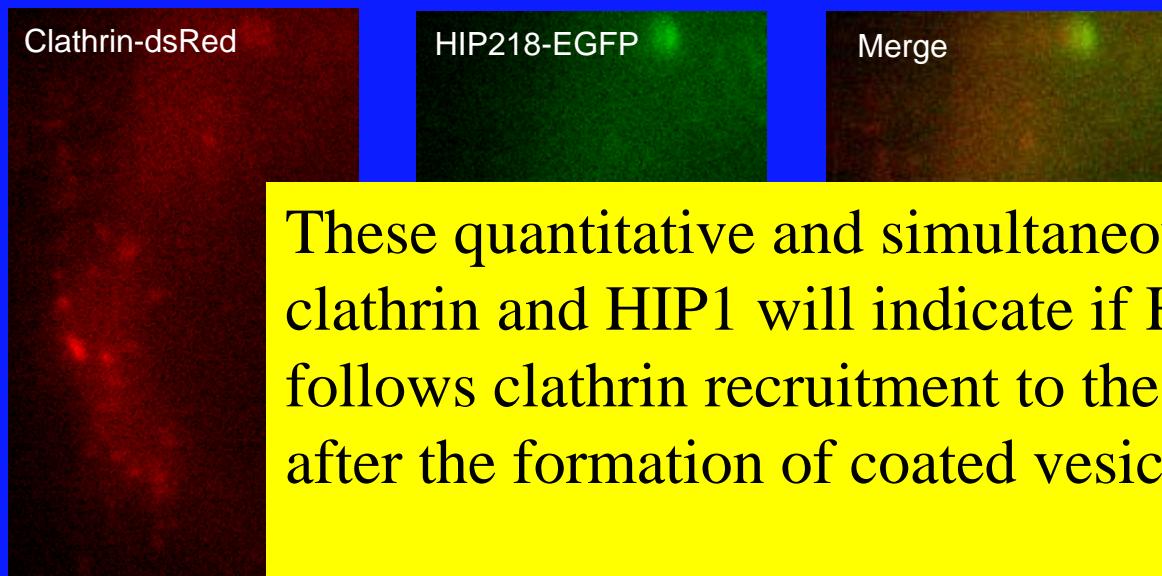
Mobility



Intensity



Dual-View Imaging



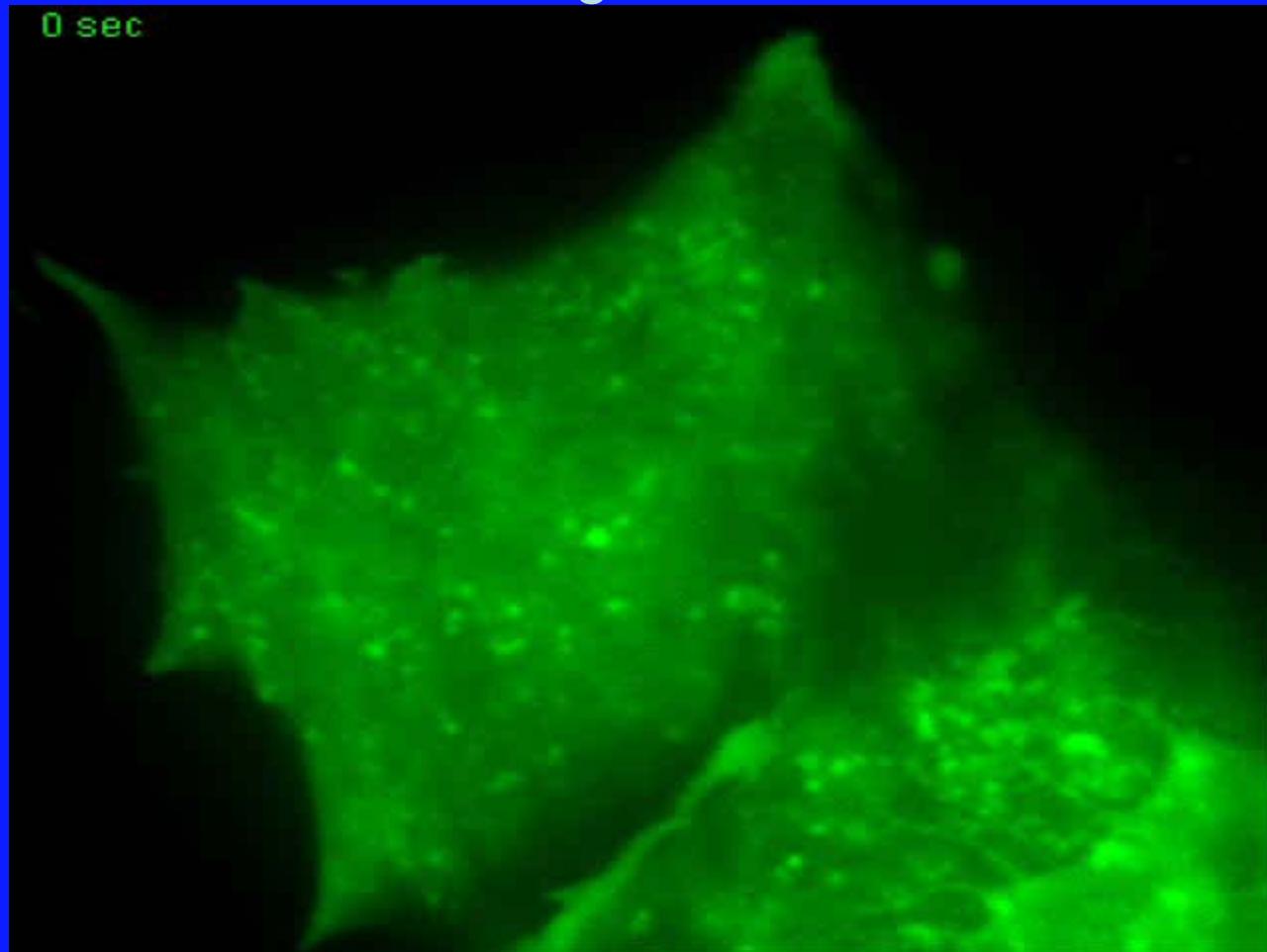
These quantitative and simultaneous measurements of clathrin and HIP1 will indicate if HIP1 precedes or follows clathrin recruitment to the CP or if it functions after the formation of coated vesicles.

QuickTime™ and a decompressor are needed to see this picture.

QuickTime™ and a decompressor are needed to see this picture.

QuickTime™ and a decompressor are needed to see this picture.

Identification of a new cytoplasmic nanoparticle, the
rasosome, as a carrier of multiple Ras molecules and its
signals



Rotblat et al. 2006

Total Internal Reflection Fluorescence (TIRF) microscopy in cell biology.

- Focus on an optical section at least 5 times thinner than confocal microscope, as fluorophores above 100-200nm range will not be excited.
- Z resolution on the nanometric scale by variation of the evanescent field depth.
 - Elimination of background fl. From outside of the focal plane-->improved signal to noise ratio
 - Improved spatial resolution

Total Internal Reflection Fluorescence (TIRF) microscopy in cell biology.

- Applications:
 - Imaging of minute structures or single molecules
 - Monitoring the interaction between intracellular protein and the substratum, focal adhesions
 - Vesicle translocation, docking, fusion and endocytosis
 - Monitoring protein-protein interaction by FRET
 - Characterization of force extracted on the substratum during cell motility
- TIRF is fast non-invasive non destructive, sensitive and versatile technique
- Can be added to conventional microscopes

Total Internal Reflection Fluorescence (TIRF) microscopy in cell biology: Drawbacks

- Restricted to one section
- Problems:
 1. Variable vesicle fluorophore content
 2. Membrane adherence to glass
 3. Uncertainty of evanescent field within cytosol
- Expression of fluorescent proteins or staining of vesicles in neurons/primary cultures (pSFV, electroporation).
- Analysis software/procedure for vesicle movements
- CCD detectors: spatial and time resolution

Many thanks

