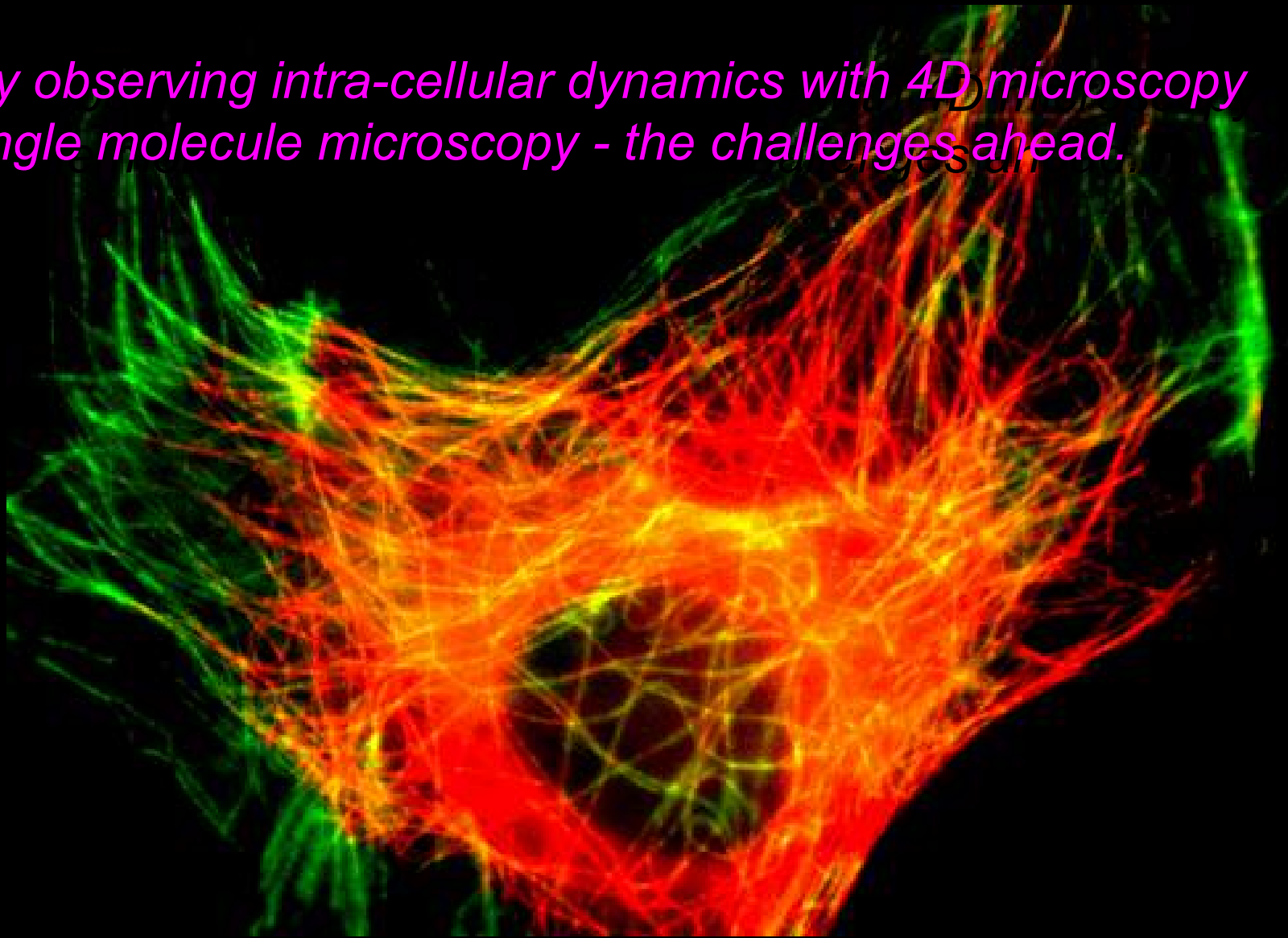


*Directly observing intra-cellular dynamics with 4D microscopy and single molecule microscopy - the challenges ahead.*



**Derek Toomre**

## 3 Aims of this talk

1. Overview of present and emerging biological applications that will require fast ultrasensitive imaging.
2. Overview of the parameters and challenges of advanced cellular imaging
3. ***Educational yet entertaining: Take YOU on an visual ride, from just barely skimming the cell surface, to deep into cells.***

# Live Cell Imaging:

A Bright Future with New Molecular Tools,  
Microscopes & Cameras to Image Cellular Dynamics

## THICK

- 4D/5D Imaging (3D+time+wavelength)

## THIN

- Total Internal Reflection Fluorescent Microscopy (TIR-FM)

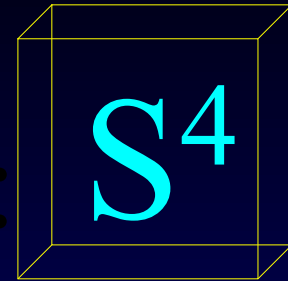
## *SINGLE MOLECULE*

- TIR-FM
- FCS

# Outline

1. 2D timelapse imaging
2. TIRFM (Thin) imaging of single vesicle and molecules
3. 3D imaging
4. 4D/5D imaging
5. Future perspectives

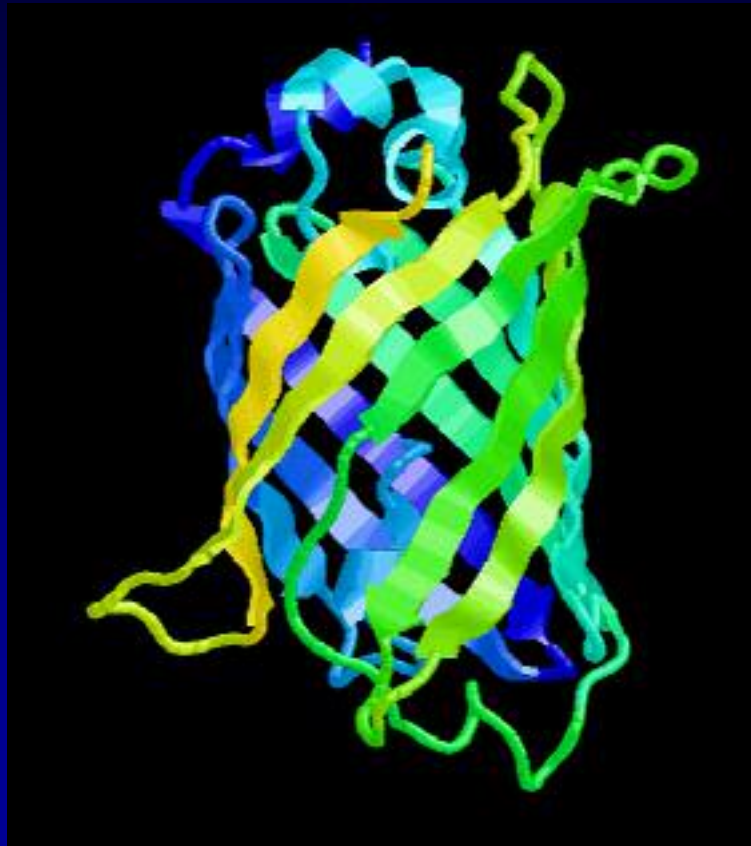
# Camera Challenges\*:



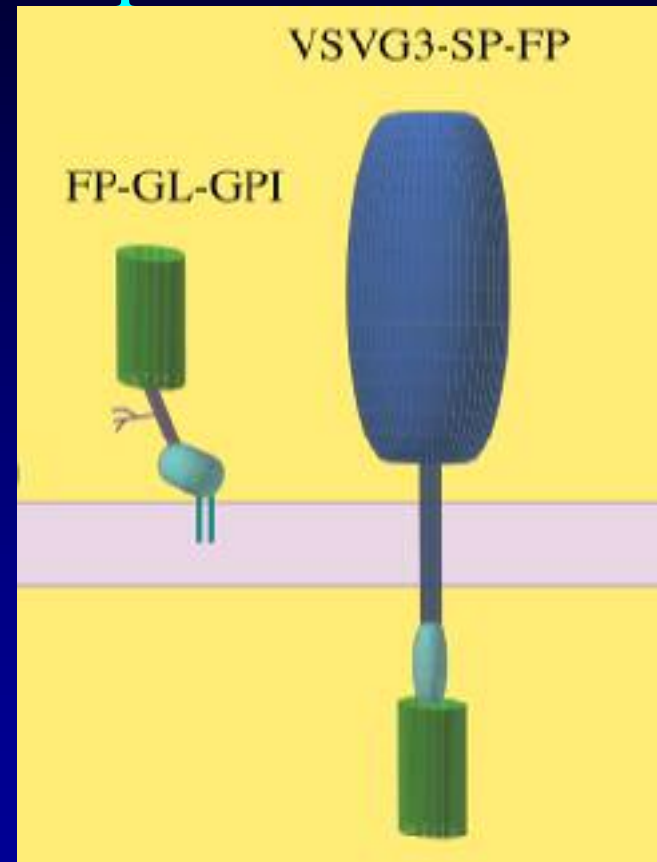
- Speed of acquisition
- Sensitivity of detection (low light)
- Signal detection efficiency (QE)
- Size of pixel / frame

\*subjective biological perspective

Live Cell Imaging: We are seeing a revolution  
in the biological sciences, the revolution is  
seeing molecules in space and time.



Green Fluorescent Protein



(X)FP=  
GFP/YFP/CFP/RFP

# 2D Timelapse Imaging

## IMPORTANT PARAMETERS

1. Speed
2. Multicolor
3. Minimal Photobleaching
4. High resolution

# Object Characteristics: Small, Fast & Dim

## Size

- Variable: 25nm - 100s nm

## Speed

- Up to 2 microns/sec! *No stargazing here...*

## Intensity

- Variable: ~10 - 1000 fluorophores/vesicle

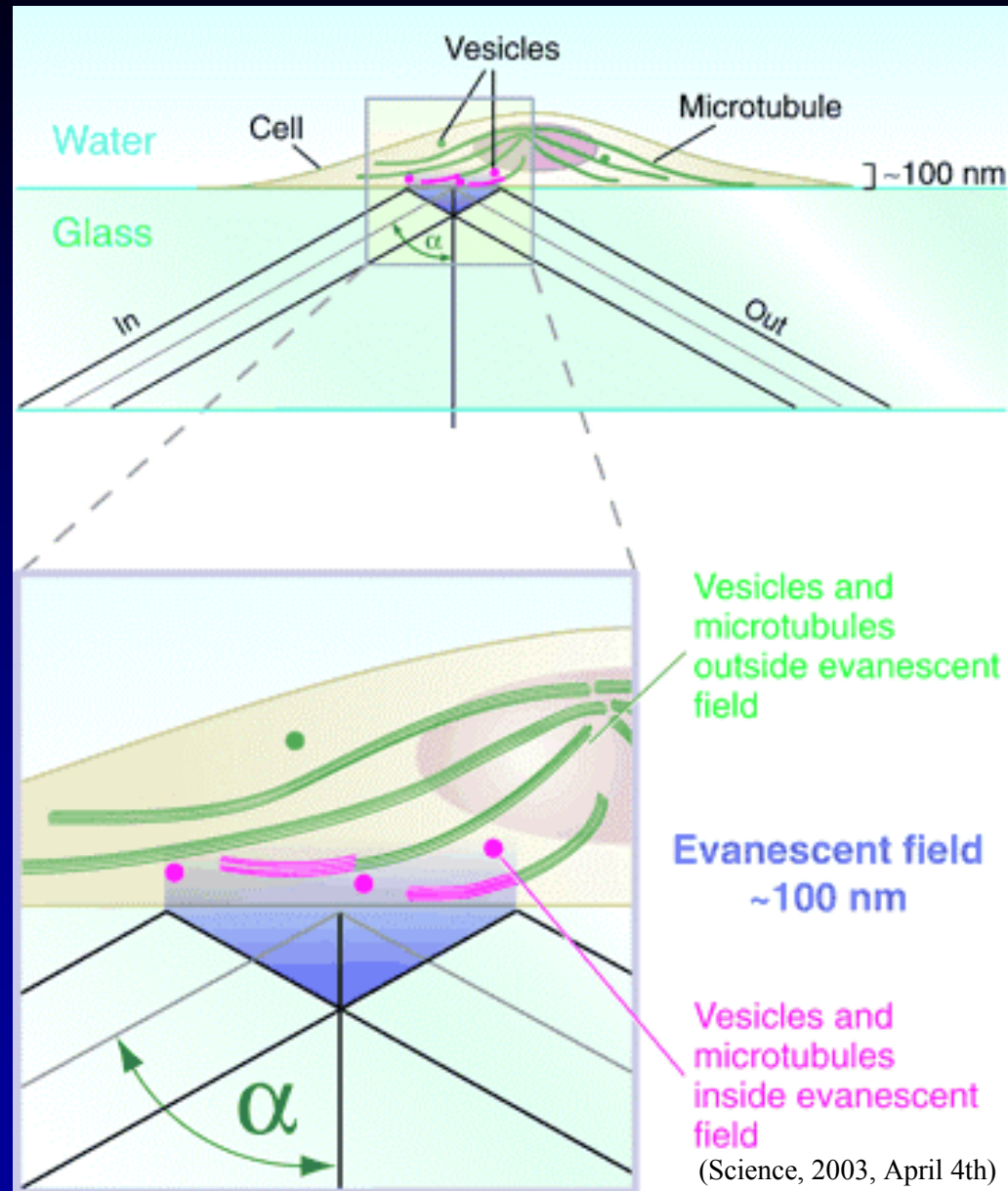
## Dynamic Range

- ~10 bit is adequate for most applications

# Total Internal Reflection Fluorescent Microscopy (TIRFM) Imaging

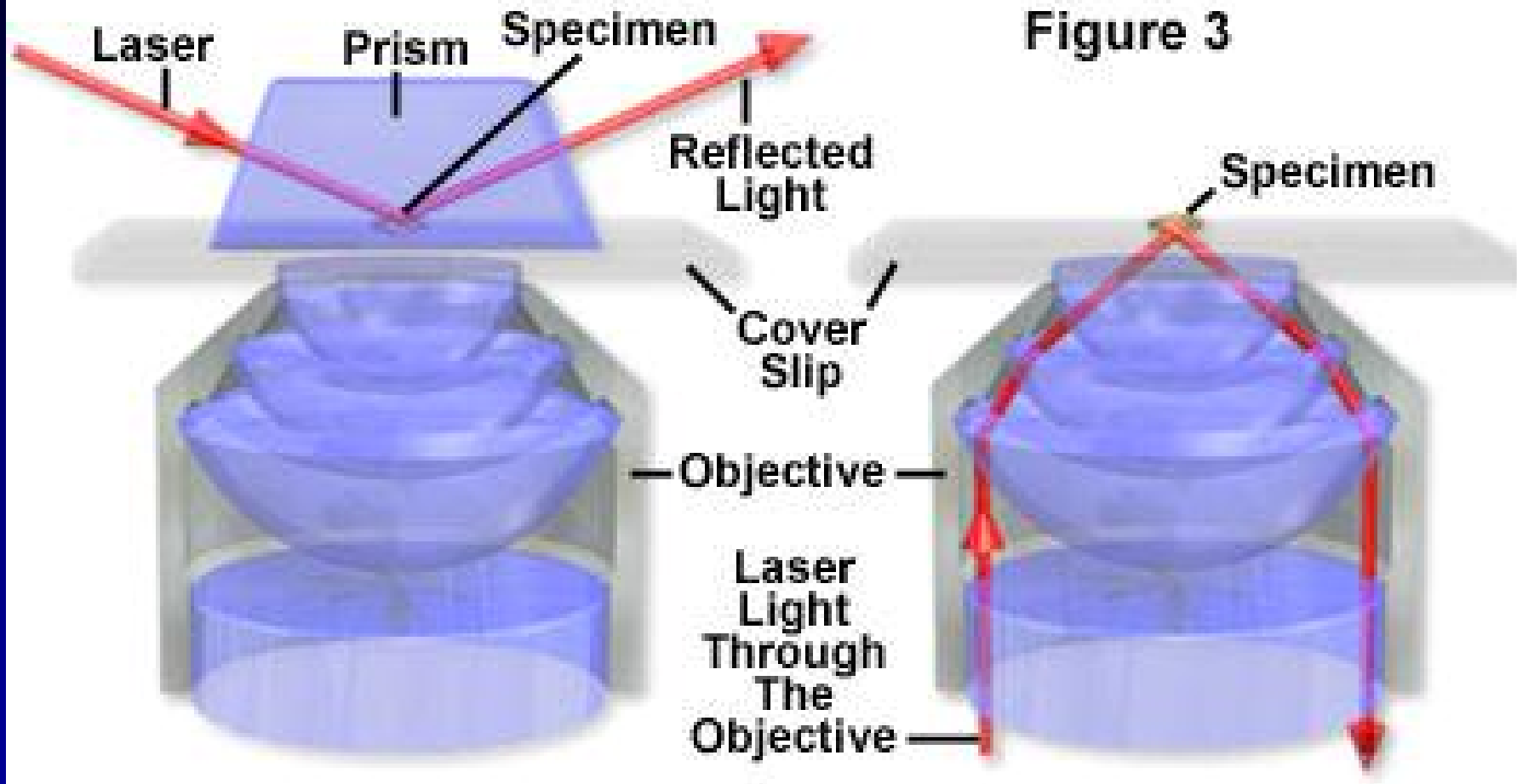
--> Vesicle Trafficking

# Total Internal Reflection Fluorescent Microscopy



# Total Internal Reflection Fluorescent Microscopy

TIRFM Instrument Configurations



# TIR-FM Advantages & Applications

## ADVANTAGES

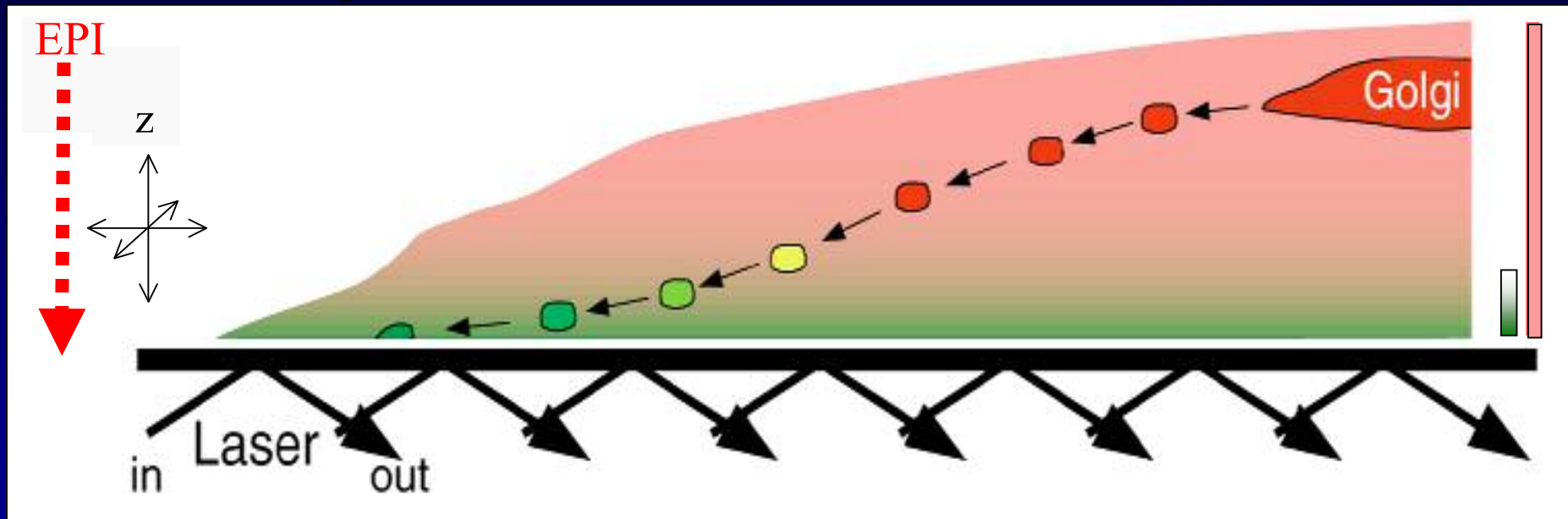
- Excitation **Light Exponentially Decreases** Away from the Coverslip - Penetrates Depth of only **~50-200nm**
  - > *Minimal Photodamage, Incredible Signal-to-Noise*

## APPLICATIONS

- Permitted Observation of Exo- and Endocytosis:
  - e.g. Chromaffin Granules, Synaptic Vesicles (Almers Lab)
- Cytoskeleton Dynamics near the Cell Surface
- Permitted Observation of Single Molecules
  - e.g. EGF and binding to its receptor (Yanagida Lab)

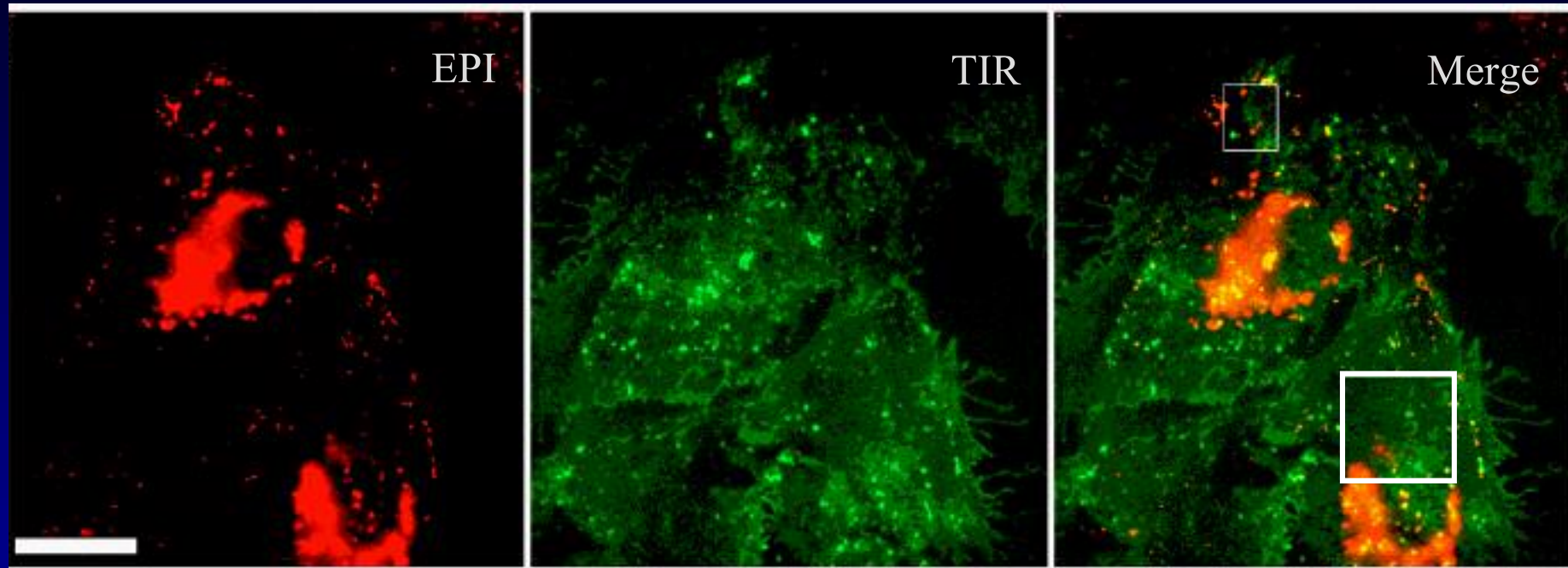
# Principle of Combined EPI & TIR

Wide Field Epi-Illumination (EPI)

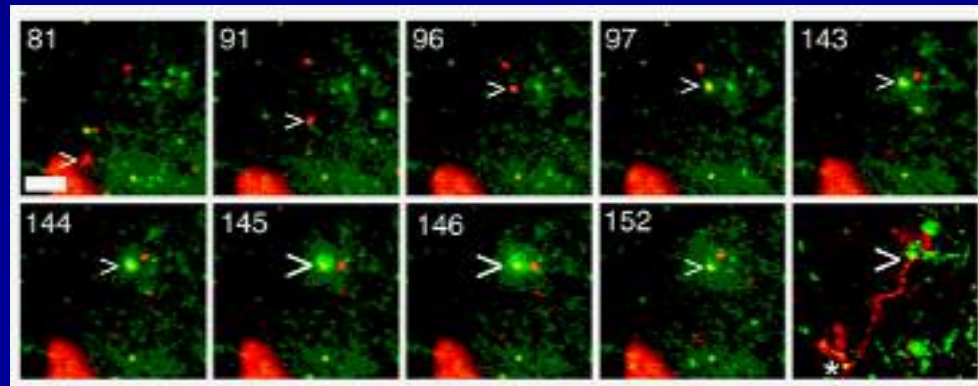


Total Internal Reflection (TIR)

# Combined EPI & TIR to Monitor the Late Constitutive Secretory Pathway

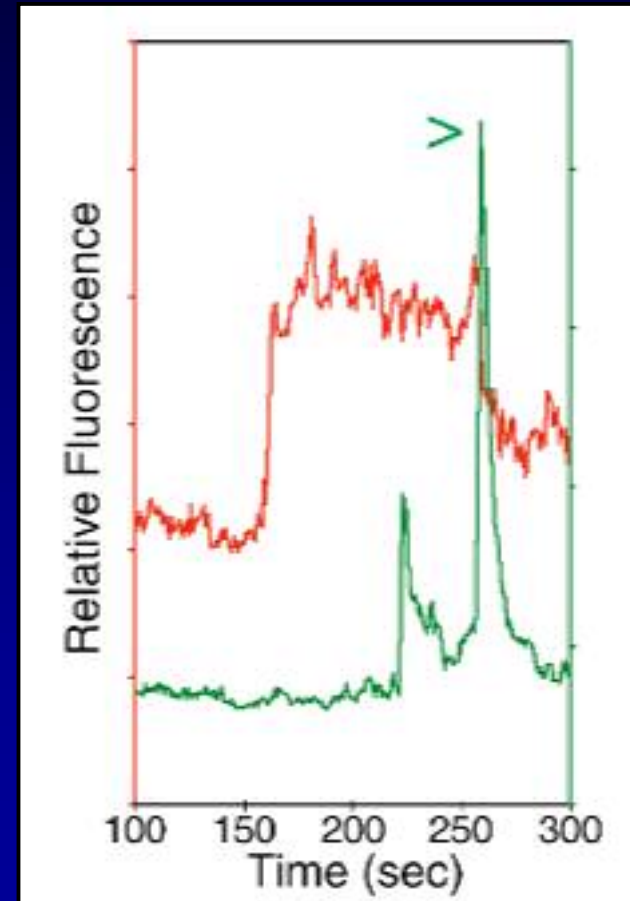
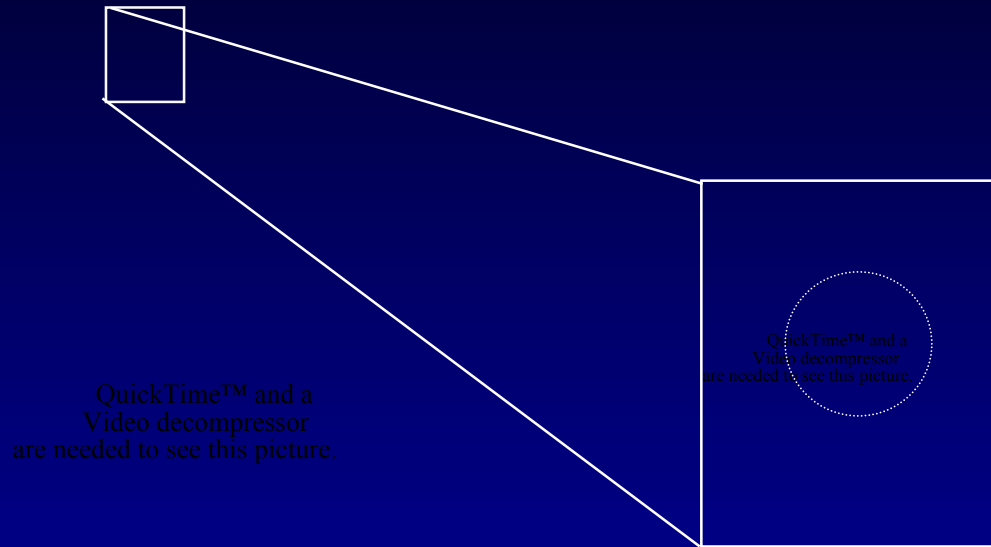


10 microns



# Dual EPI and TIR

- VSVG3-SP-YFP
- EPI & TIR
- Acquire 1fps, play 12x

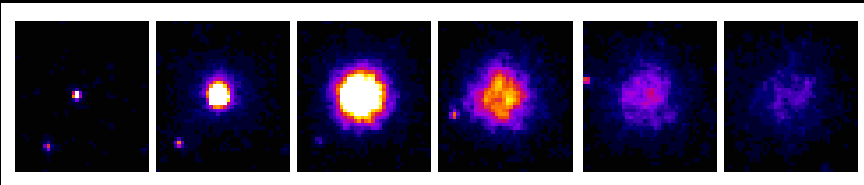


[Toomre et al., JCB., 2000]

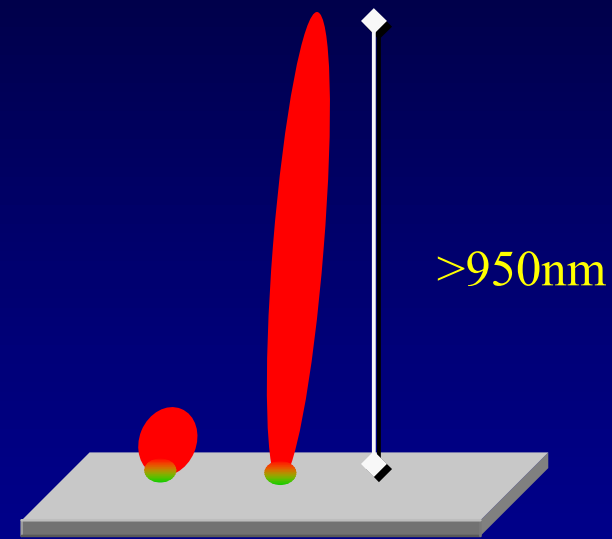
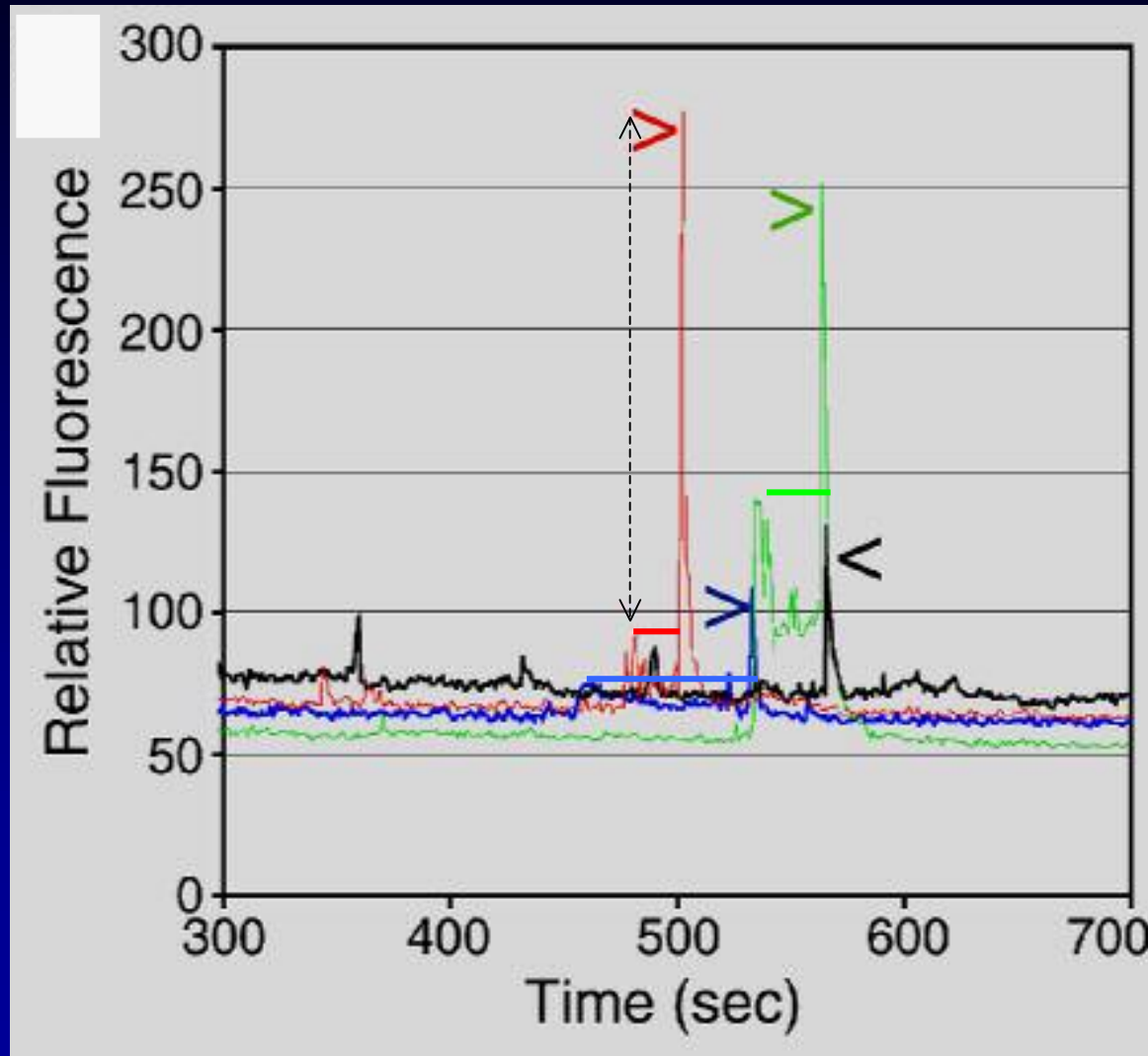
# Fusion of Constitutive Cargo

- TIR
- VSVG3-SP-YFP in PtK2 cells
- Acquire 1 fps, Play 15 fps (15x)

10 microns



# Analysis of “Docking” Prior to Fusion



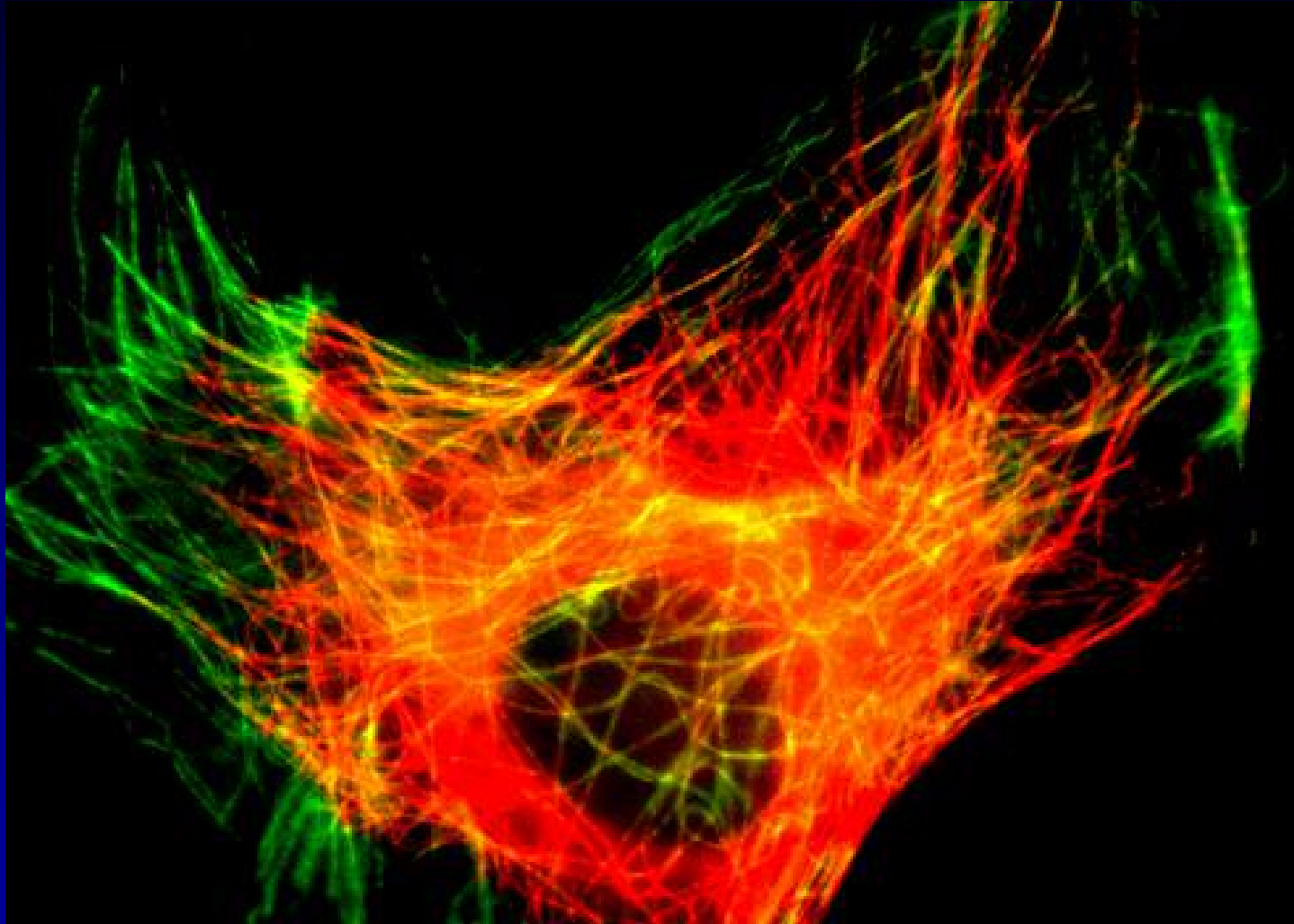
- 1) Relative Size of Carriers
- 2) Measure “Docking” Time

# TIRFM & Cytoskeleton

## Two Views of Tubulin-YFP (PtK2 cells)

TIR

EPI



# Single Molecule TIRFM Imaging

# Single Molecule:

## Examples of Biological Applications

1. Tracking Motors (Kinesin, Myosin)
2. Tracking Movement of proteins in the membrane (e.g. Lipid Rafts)
3. Observing Virus Trafficking
4. Imaging Signal Transduction (Spatial-Temporal Information)
5. Use in Combination with other Biophysical Approaches (AFM, Optical Tweezers...)

---->Single molecule measurements can reveal events not detected by ensemble-averaged detection.

# Important Factors in Single Molecule Detection

## **SIGNAL/NOISE (S/N)!!!!**

### Increase Signal

-> Choice of Fluorophore

– QE, Ext. Coef., Photobleaching, Blinking

-> Cameras with high quantum efficiency

### Decrease Noise

-> Limit excitation (TIRFM), avoid autofluorescence

-> Ultrasensitive cameras (minimal read noise and dark current, running under operating conditions; e.g. 30-100fps)

### Information Sought?

-Position/Tracking, or Presence of dye in a spot?

**Note:** Entirely new approaches possible: **Quantum dots?**

# 3D Imaging

# 3D Stack & Deconvolution: Dual FP Markers in MDCK cells

YFP-GL-GPI

VSVG3-SP-CFP

3D Stack: raw data

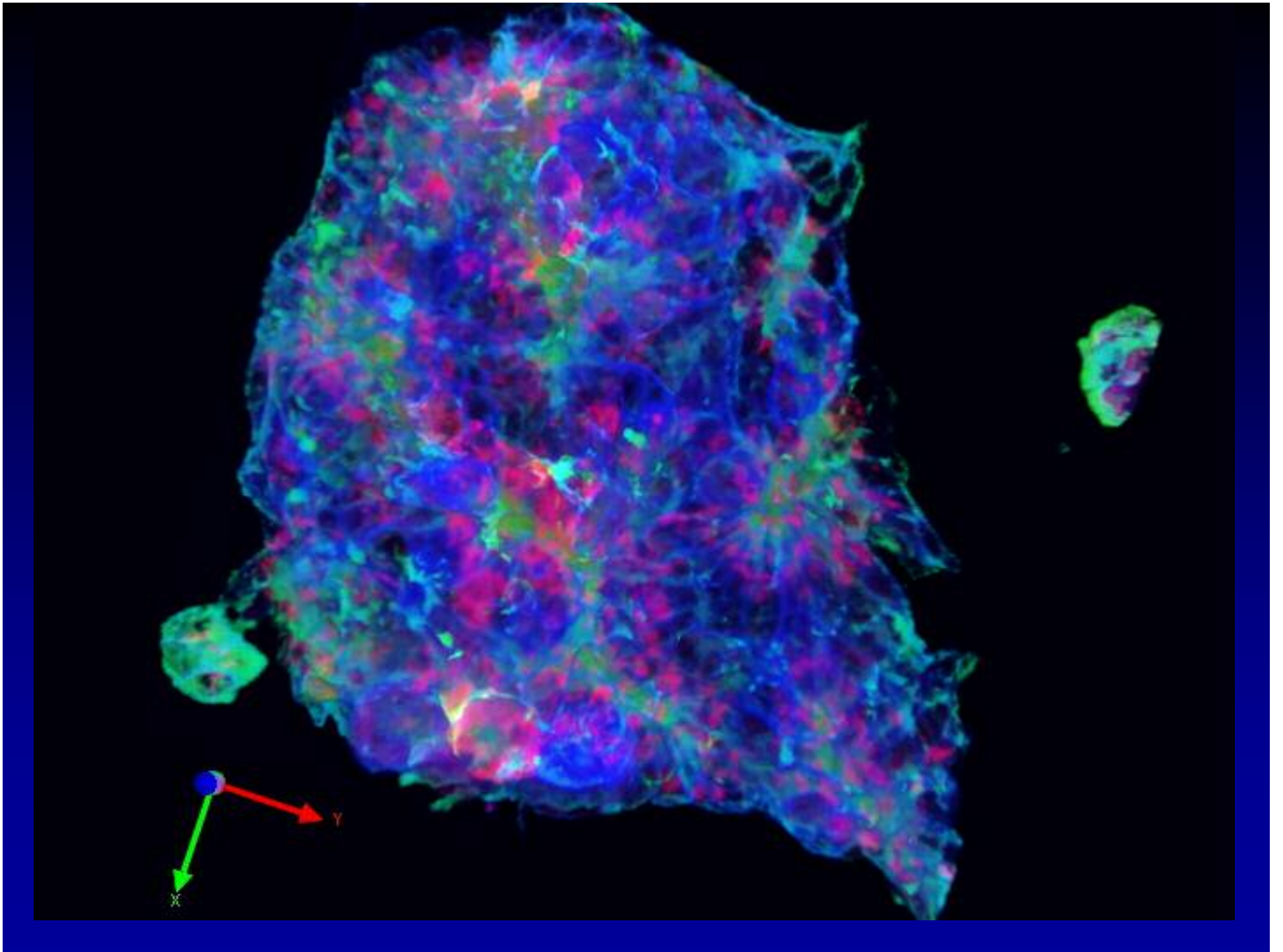
Deconvolve & Render

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

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

Filter  
←

EPI + deconvolution



# 4D/5D Imaging of cells and tissue

# Rendered Tubulin-YFP 4D stack

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

Comment: Photobleaching is a problem with Orca II CCD chips

# SUMMARY: Why are fast, sensitive and efficient detectors needed?

1. Photobleaching (general)
2. Low signal/noise (single molecules)
3. Speed:
  - Fast processes in 2D + time: eg. Calcium signals
  - Fast processes in 3D (+ time): e.g. Vesicles

# Future Cameras Needs??

SMALLER, FASTER, CHEAPER...?.

1. **Smaller Pixel Size:** Often need to image near the Nyquist Limit (but notable exceptions..).
2. **Faster SPEED:** Especially for 4D/5D imaging.
3. **Better Quantum Efficiency - EVERY PHOTON IS SACRED** -> back illuminated chips.
4. **Good Interfacing** with other hardware and controllers.

# Camera Challenges:

## My Wish List and Comments....



- Speed of acquisition: 30-60 fps for a 1 Megapixel Chip & 100 fps+ for a 25% AOI
- Sensitivity of detection (low light): EMCCD (but without clock noise)
- Signal detection efficiency (QE): 80%+ from 400nm - 650nm (most biological imaging)
- Size of pixel / frame: ~1000 x 1000 pixels (1 Megapixel) at ~6-8 micron pixel. User defined (any rectangle) AOI.
- Dynamic Range: 10-12 bit is adequate for most samples
- Software/Hardware: **Need drivers!!** Must be able to use other hardware and software (yet maintain features). Biologists would like to use the camera with THEIR system (special hardware, controllers, programs..)

Comment: Huge potential market in 4D/5D spinning-disk confocal imaging

# ACKNOWLEDGEMENTS

## YALE

- Roberto Zoncu
- Sead Begovic
- Cecile Chalouni
- Tom Gniadek

## Focal Adhesions

- Vic Small
- Olga Krylyshkina

## EMBL/MPI

- Kai Simons\*
- Patrick Keller\*
- Elina Diaz\*
- Dietmar Manstein

## SUPPORT

- Olympus / Till Photonics / Zeiss / Andor Technologies
- Ludwig Institute for Cancer Research Foundation (LICRF)

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August 2, 2001

**YALE UNIVERSITY  
SCHOOL OF MEDICINE**

