

# An Investigation of Clathrin-Mediated Endocytosis through Three-Color Super-Resolution Microscopy

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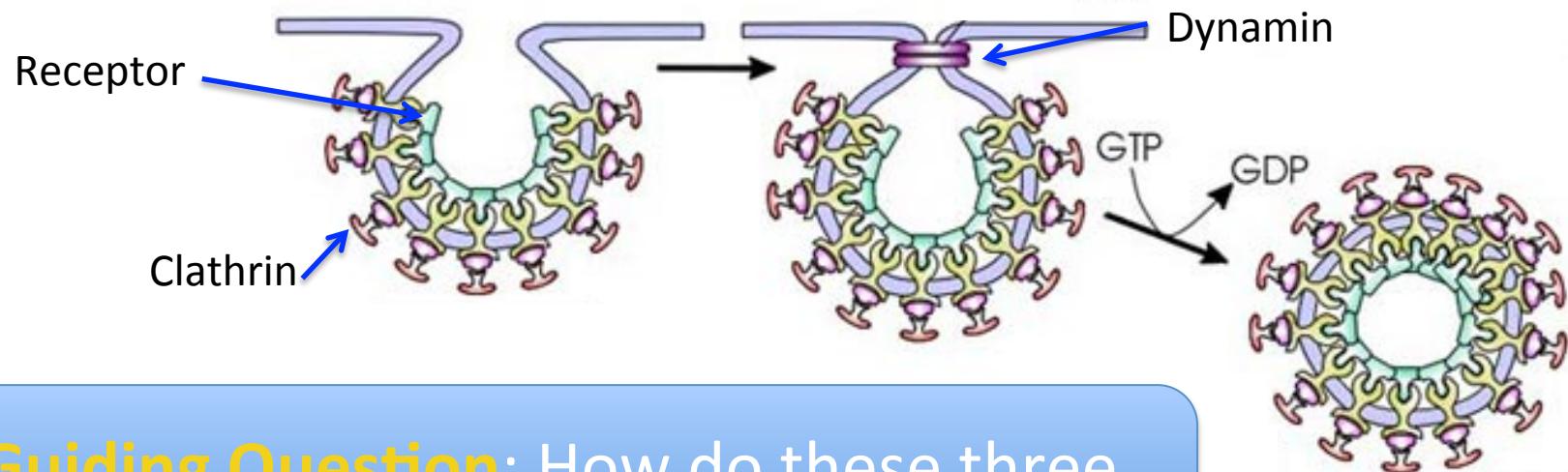


# BACKGROUND



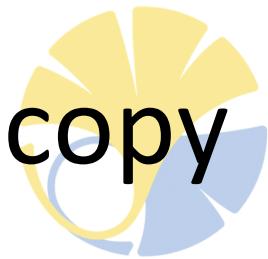
# Clathrin-Mediated Endocytosis

- Endocytosis: the process by which extracellular cargo is engulfed by the cell and shuttled to cytoplasmic endosomes.
- Putative mechanism:
  - Cargo molecules bind to the appropriate **receptor** on the plasma membrane
  - A **clathrin coat** forms on the cytosolic surface, inducing membrane curvature
  - **Dynamin** assembles at the neck and induces vesicle scission

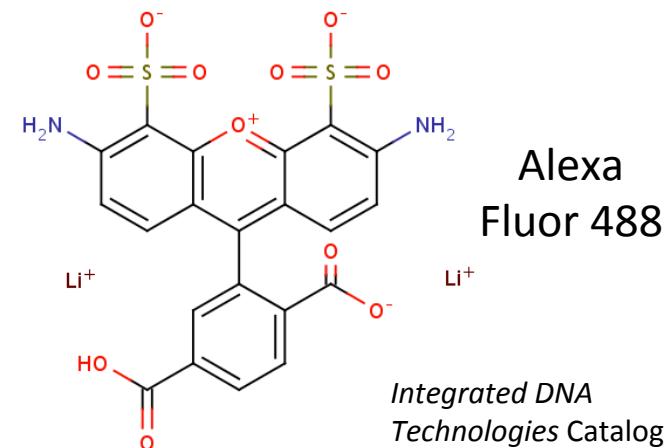
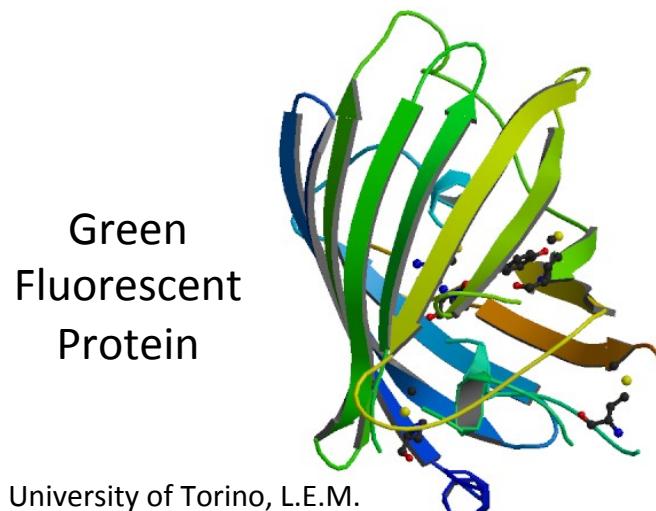


**Guiding Question:** How do these three proteins interact to facilitate endocytosis?

# Multi-Color Fluorescence Microscopy



- Fluorescence microscopy: exploits the inherent fluorescent properties of certain molecules to label and track molecules of interest.
- Provided the emission channels are spectrally distinct, we can, in theory, take simultaneous images of an arbitrary number of fluorophores.
- Fluorescent Proteins

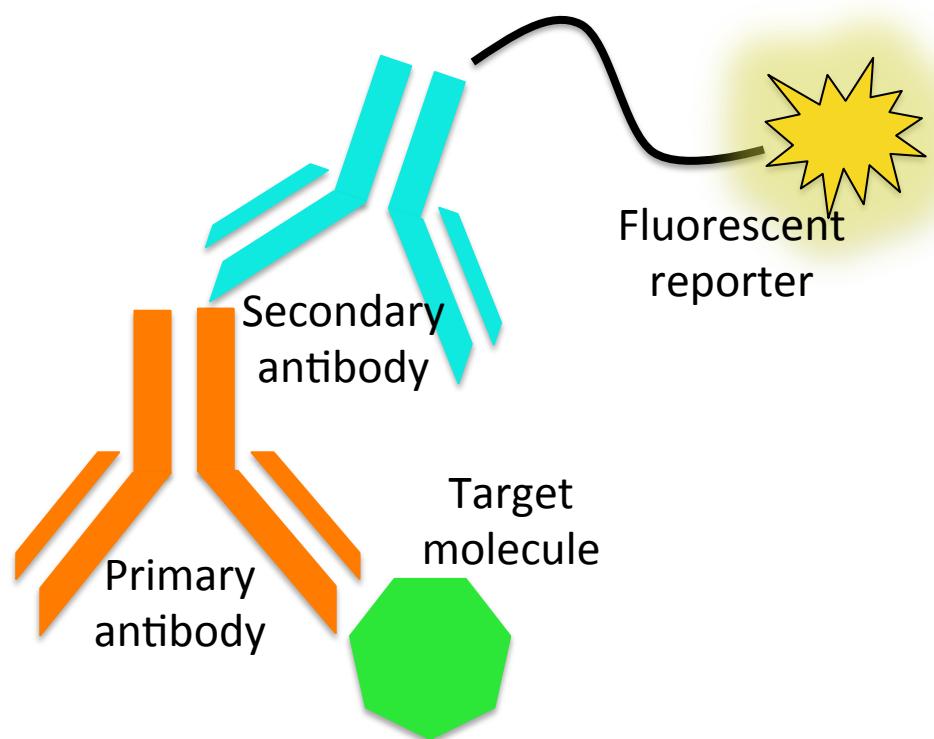
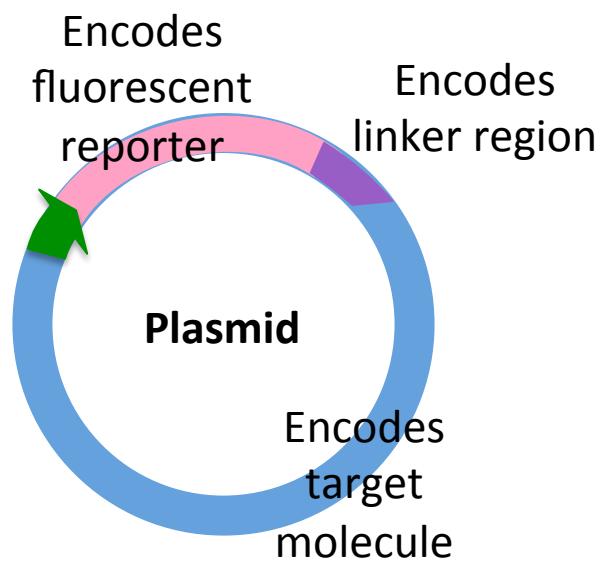


- Fluorescent Dyes



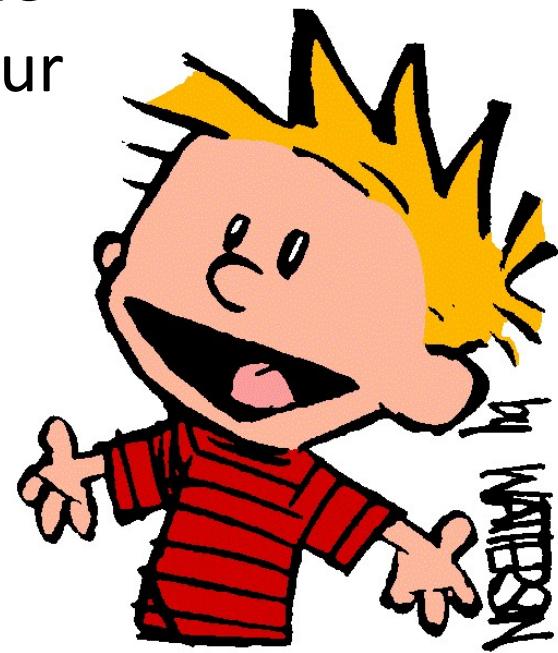
# Fluorescent Labeling Strategies

- With fluorescent proteins: generate chimeric proteins (fluorophore + target molecule) that can be transfected and expressed in cells
- With fluorescent dyes: conjugate the fluorophore to a secondary antibody, which associates with a primary antibody attached to the target molecule *in situ*





Great! So let's just attach fluorescent probes to our target molecules, and then the whole subcellular regime will be at our fingertips, right?



If only it were that simple...



# The Diffraction Limit

- Light is a wave, and thus subject to wavelike phenomena.
- Therefore, when an electromagnetic ray strikes an object, it diffracts slightly around the edges; rather than observing a single point, we see a intensity distribution.
- When two points are close to one another, their intensity distributions overlap, and we cannot resolve the individual sources.



$$R = \frac{0.61\lambda}{NA}$$

Wavelength of Emission light



Northern Arizona  
Astrobiology Club

Therefore, it would appear that our ability to arbitrarily magnify an image is **FUNDAMENTALLY LIMITED** by the wavelength of visible light .

# Super-Resolution Microscopy via PALM



- Due to the diffraction limit of visible light, the best conventional fluorescence microscopy can achieve is a lateral resolution of 200-300 nm and an axial resolution of 500-700 nm.
- **PALM: Photoactivated Localization Microscopy**
- Basic Strategy:
  - Observe the fluorescence of a few particles at a time, provided they are separated by a distance larger than the diffraction limit.
  - Fit each diffracted spot to a Gaussian distribution, thus estimating the centroid location.
  - Repeat this process for ~10,000 frames and superimpose all the processed frames to generate the final image.

$$\Delta_{loc} \approx \frac{\sigma_{PSF}}{\sqrt{N}}$$

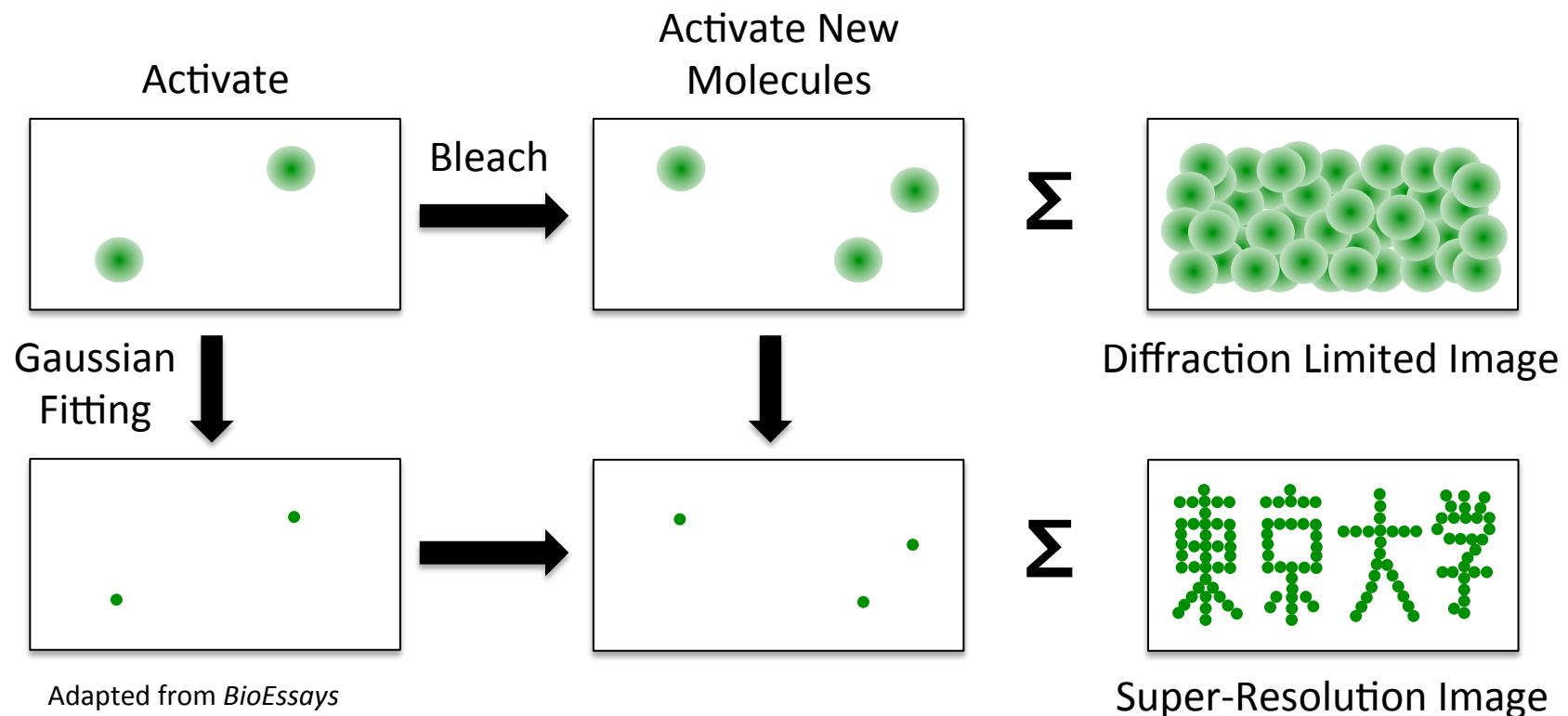
Number of  
photons detected

**The Upshot:** Resolution is now limited by number of photons emitted, NOT by incident wavelength.

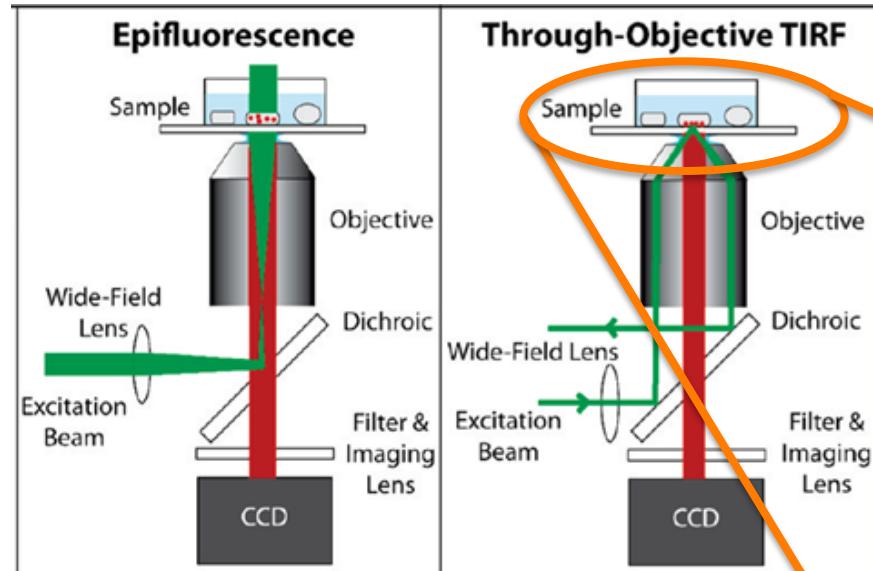
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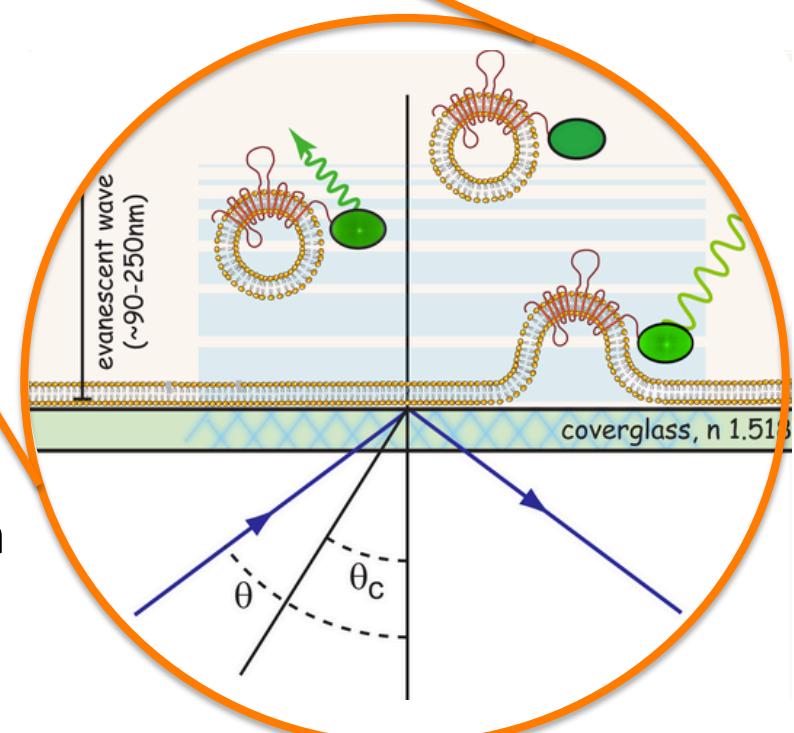


# Total Internal Reflection Fluorescence (TIRF) Microscopy



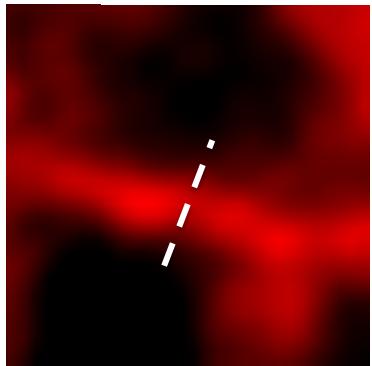
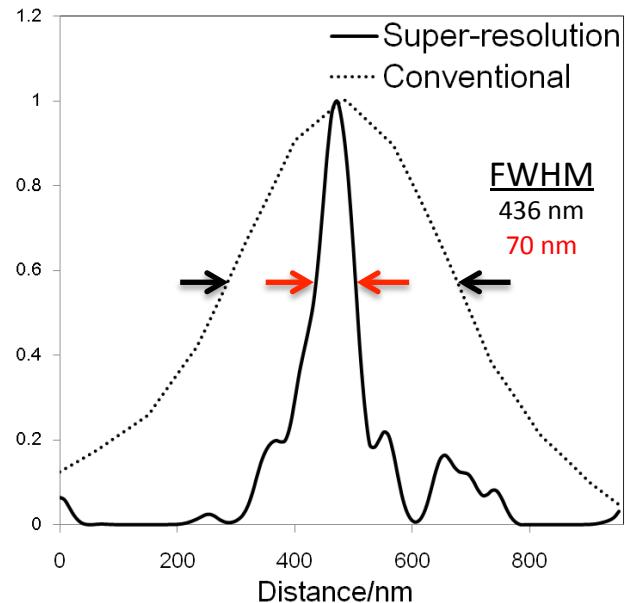
*Analytical Chemistry, 82 (6), 2010*

TIRF Microscopy allows for **single-molecule imaging**: a requirement for super-resolution microscopy

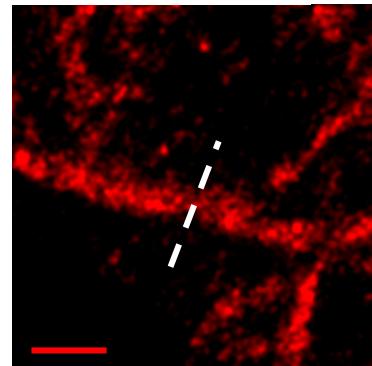


Under TIR conditions, an evanescent wave is produced whose *intensity decays exponentially* with distance from the incident plane. Thus, fluorophore excitation is induced in a very thin slice of sample (100-150 nm).

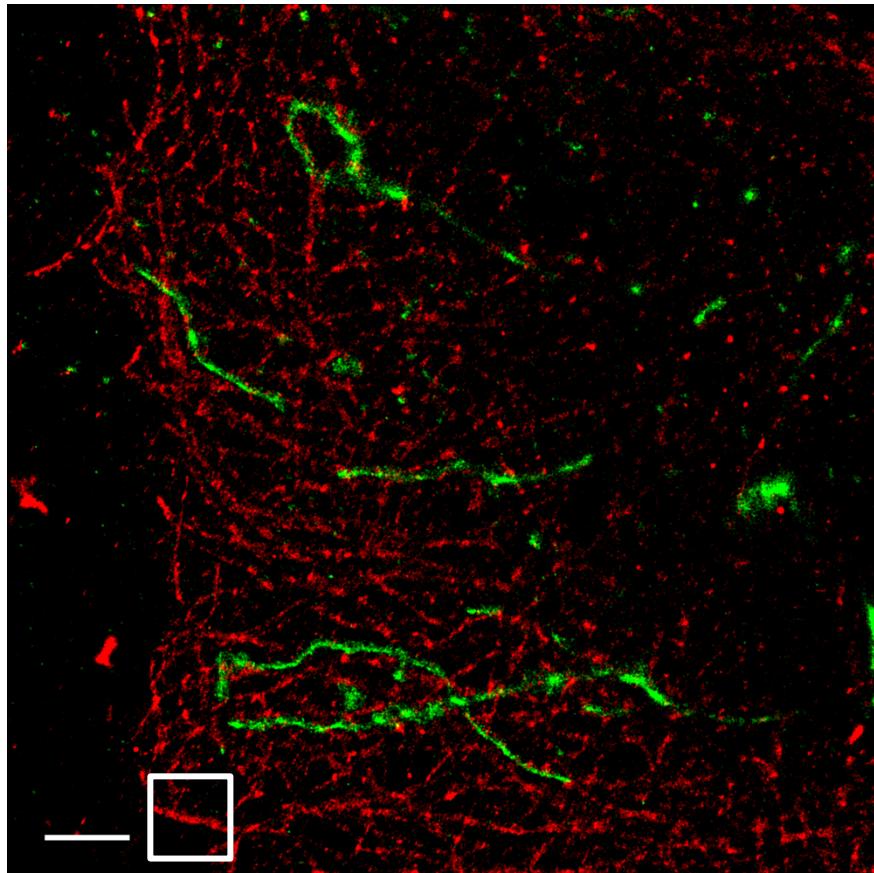
# The Power of Super-Resolution Imaging



Conventional  
Microscopy



Super-resolution  
Microscopy with  
TIRFM and PALM



Super-resolution microscopy  
**breaks the diffraction limit!!!**



# RESULTS AND ANALYSIS



# Experimental System

Three Constructs (expressed in COS-7 cells)

**EYFP—TfR**

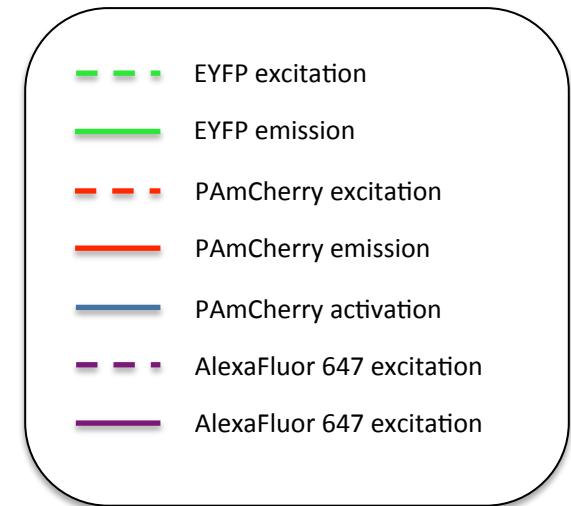
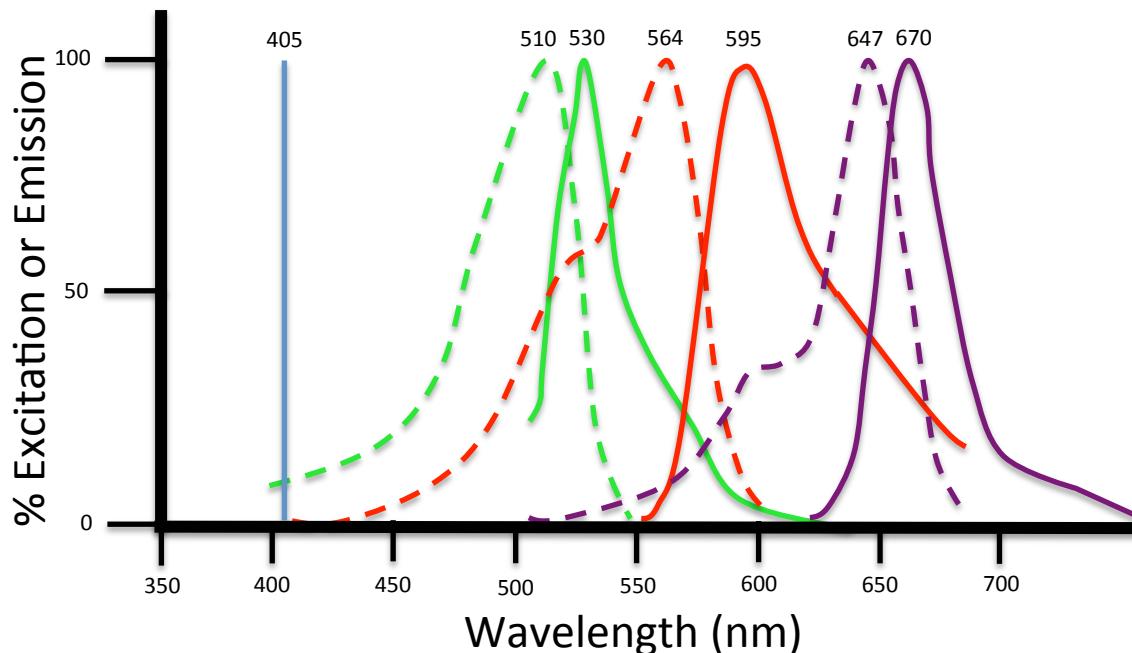
**PAmCherry—Clathrin**

**AlexaFluor 647—Dynamin**

Genetic Expression

Antibody conjugation

## Spectral Details:

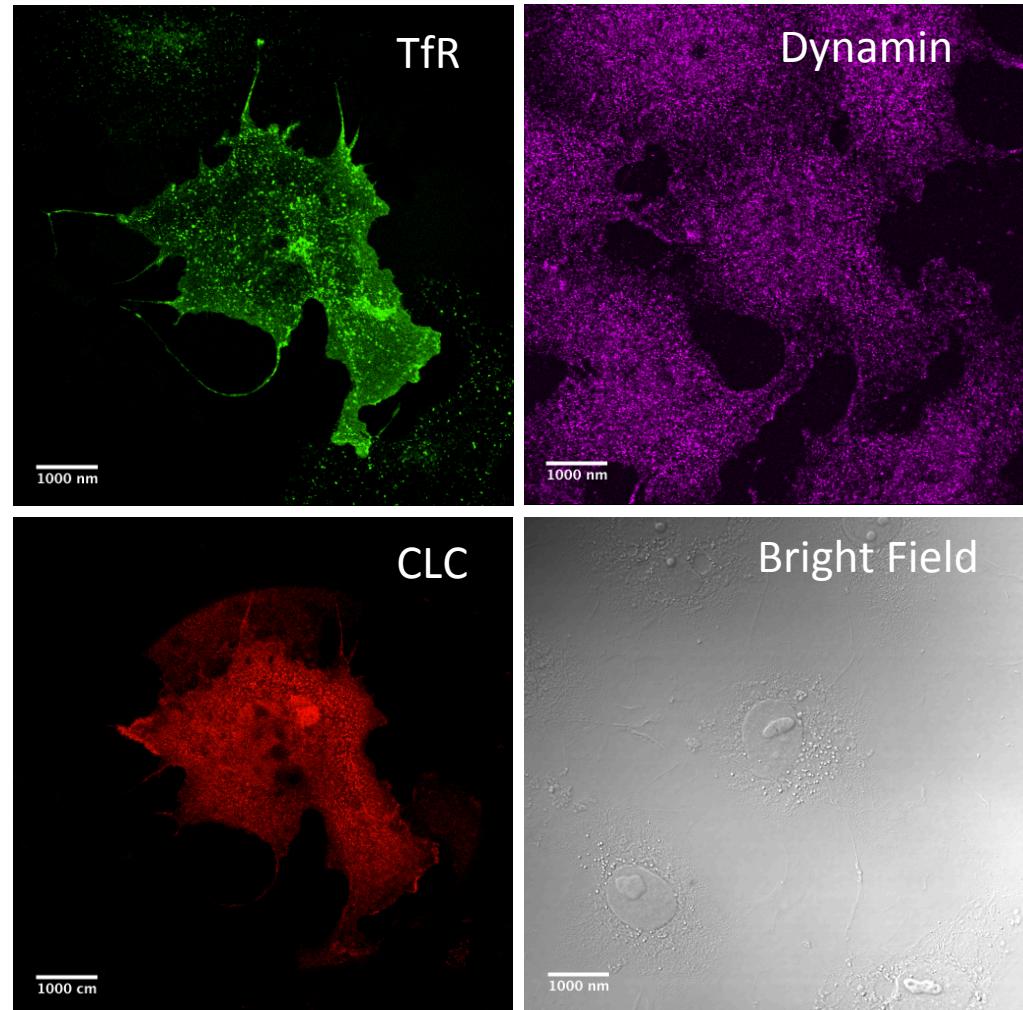




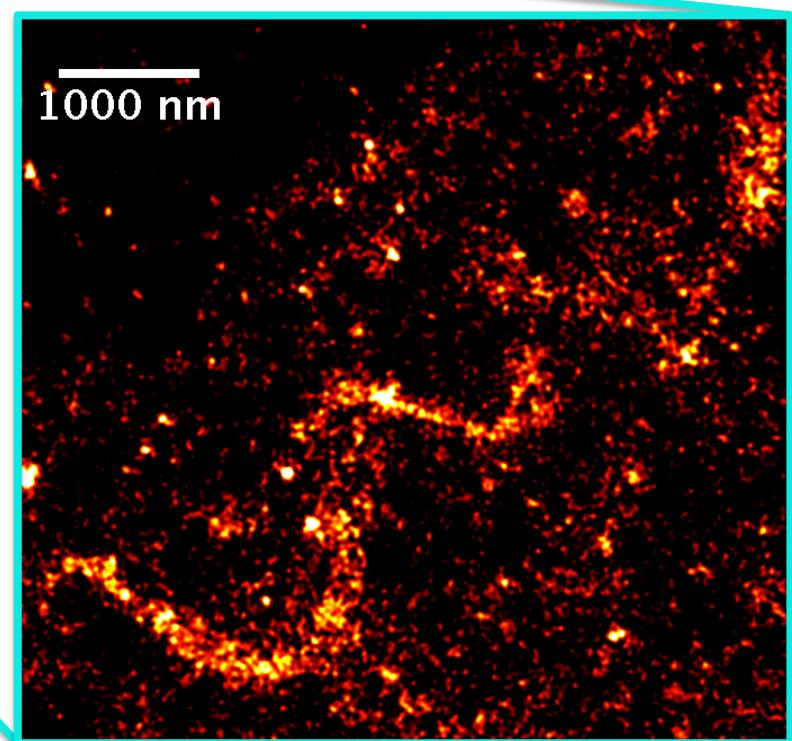
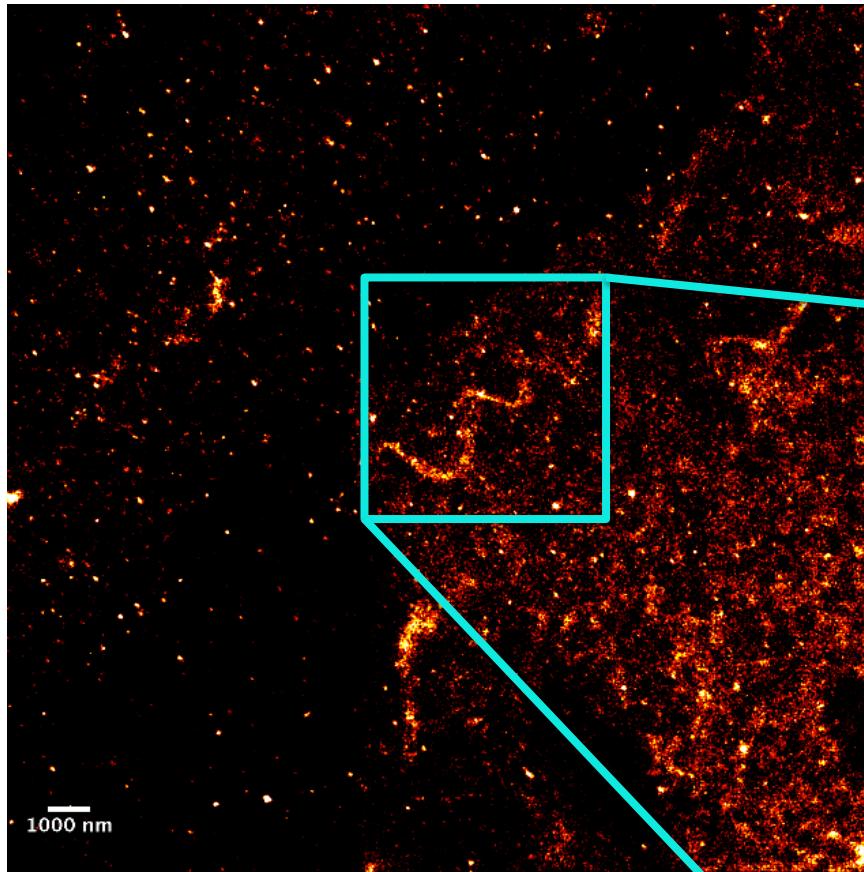
# Confocal Microscopy

- Confocal Microscopy: a wide-field imaging technique that operates on the principle of point-illumination.
- Serves as a means of validating our experimental approach:
  - Were the fluorophores properly expressed?
  - Does the photoactivation mechanism work as expected?
  - Do the cells exhibit reasonable morphology?

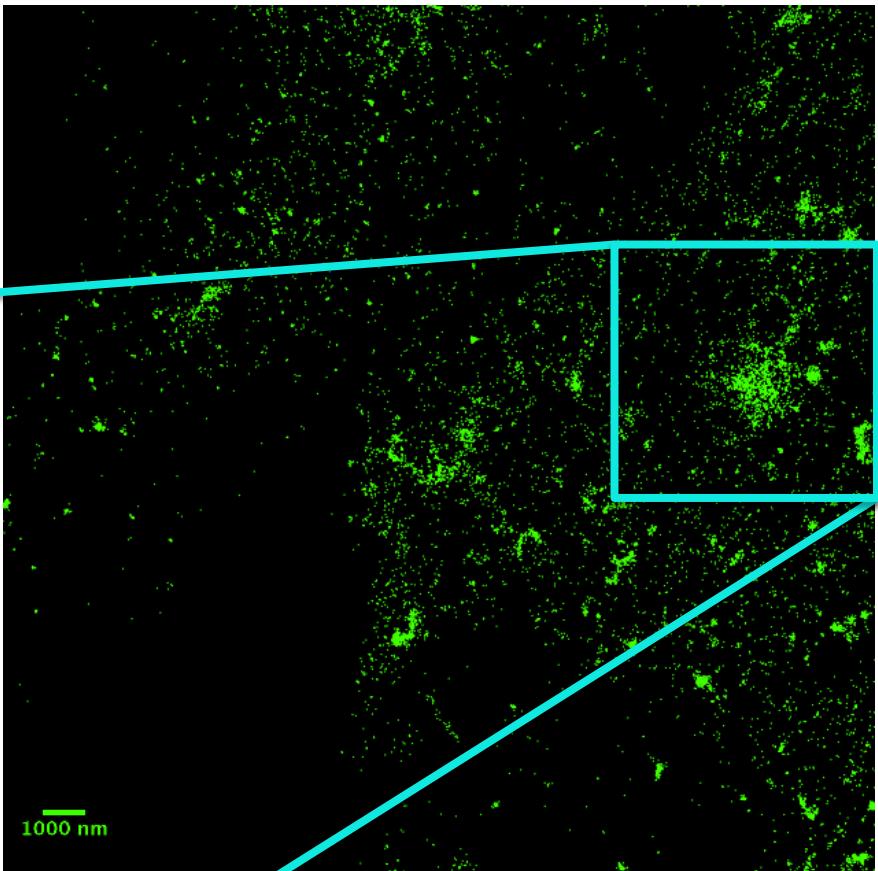
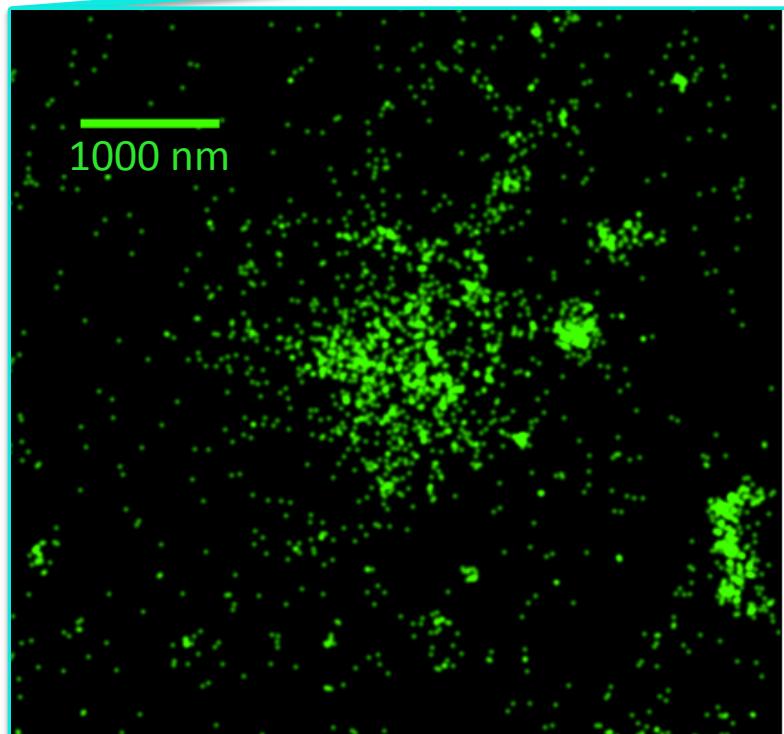
Target molecule labeling: **confirmed**.



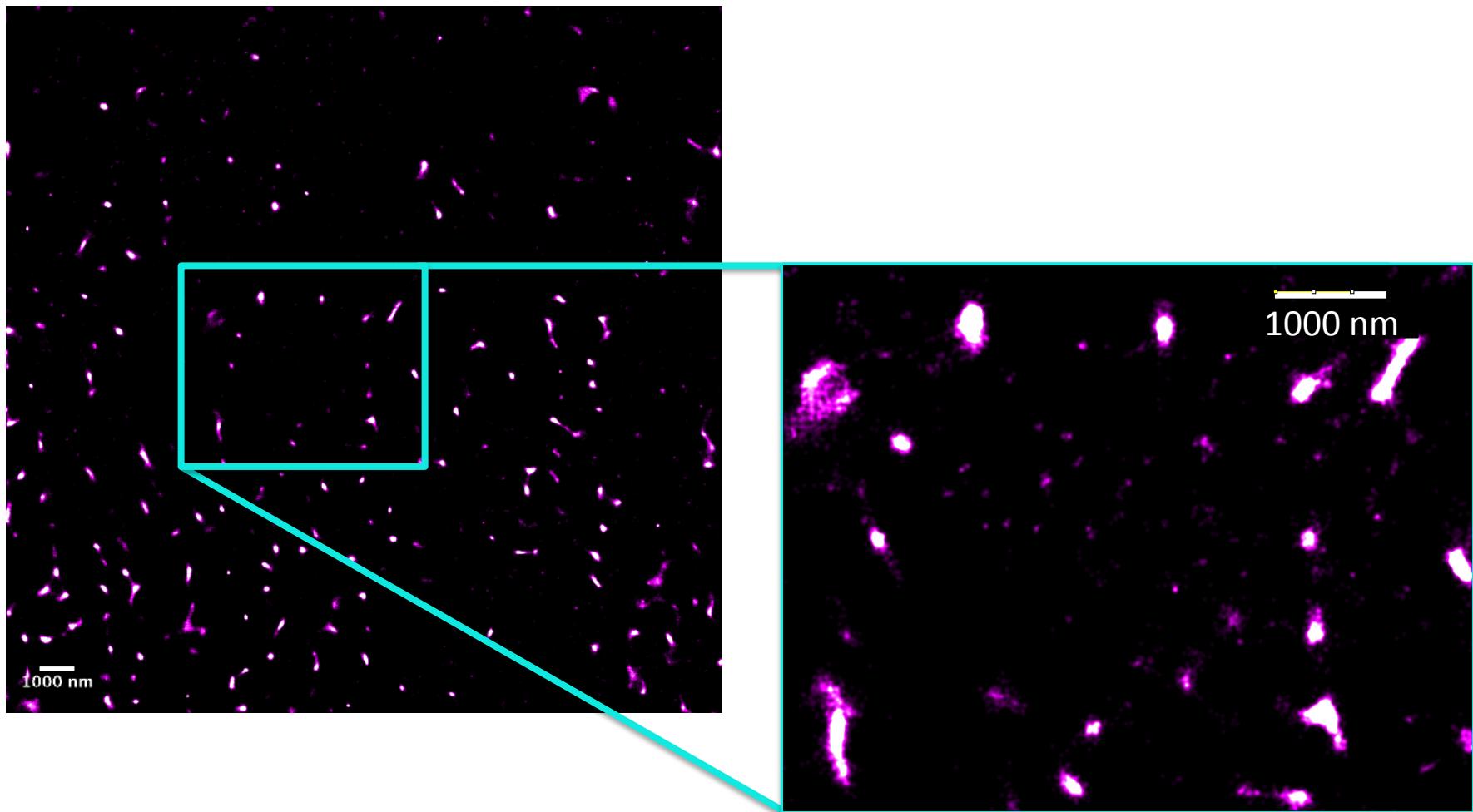
# Super-resolution: Clathrin Light Chain Clusters



# Super-resolution: Transferrin Receptor Clusters



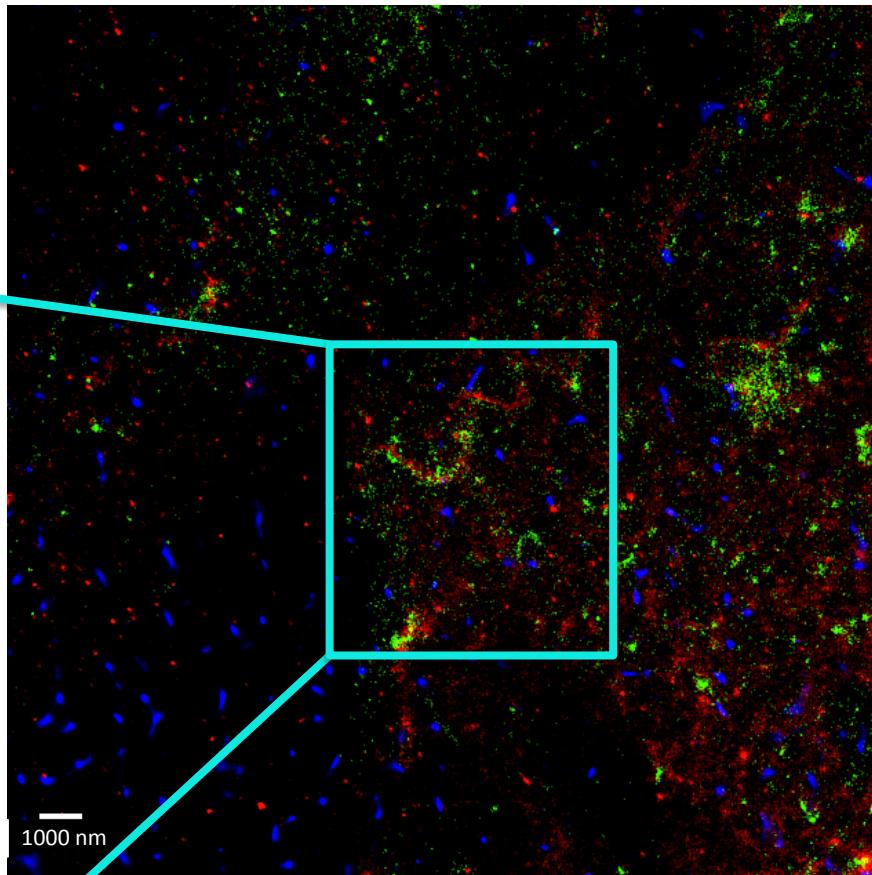
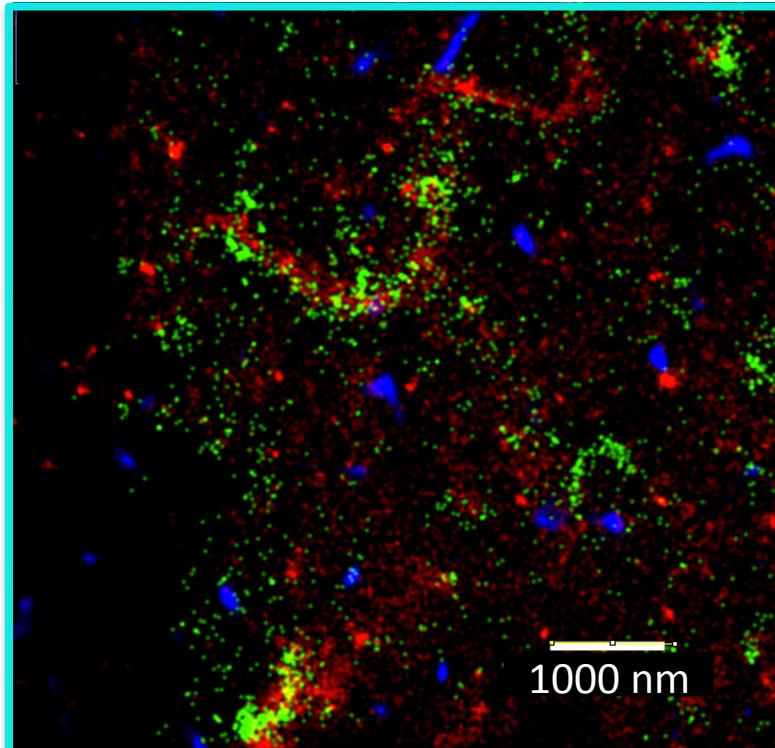
# Super-resolution: Dynamin Clusters





# Colocalization

Red: CLC  
Green: TfR  
Blue: Dynamin





Hobbes is  
such a killjoy.

Wow!  
There seems to be a lot  
of clustering and  
colocalization going on!  
So we're done, right?

Qualitatively, the results  
corroborate our expectations.

However, more rigorous  
analysis is required to yield  
truly meaningful data.

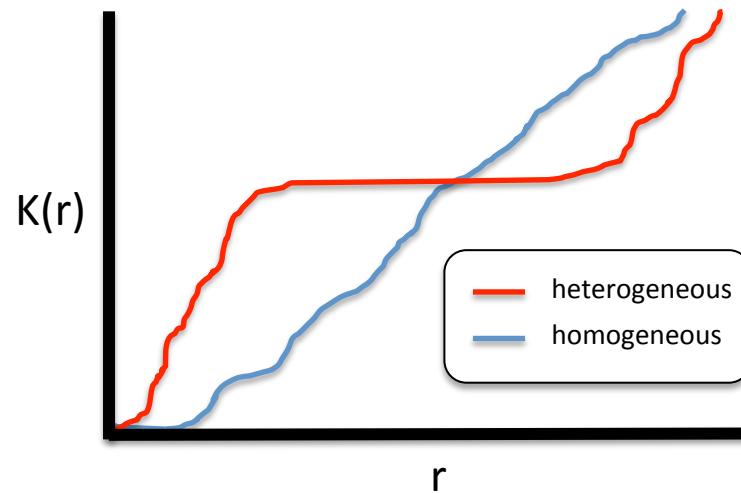
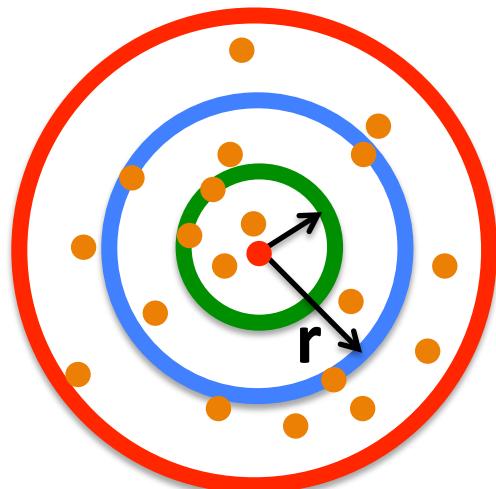




# Ripley's K Function

- A method for quantifying point process data's deviation from spatial homogeneity.
- Plots distance  $r$  versus the number of particles that fall within a circle of radius  $r$  for a given particle.

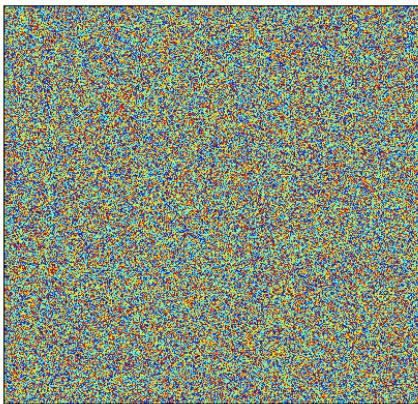
$$K(r) = \frac{A}{n^2} \sum_{i=1}^n \sum_{j=i+1}^n \delta_{ij} \quad \text{where } \delta_{ij} = 1 \text{ if } \delta_{ij} < r \\ \text{and } \delta_{ij} = 0 \text{ otherwise.}$$



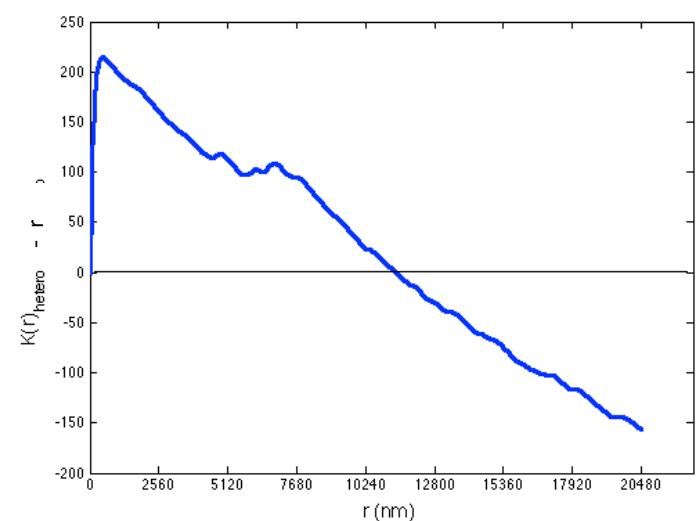
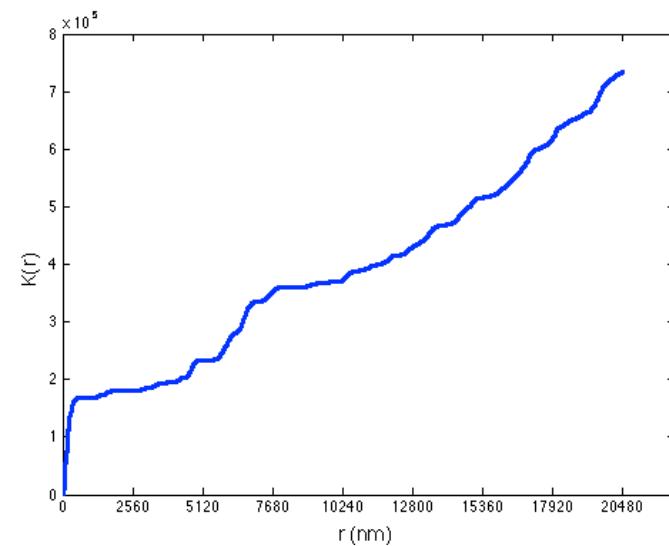
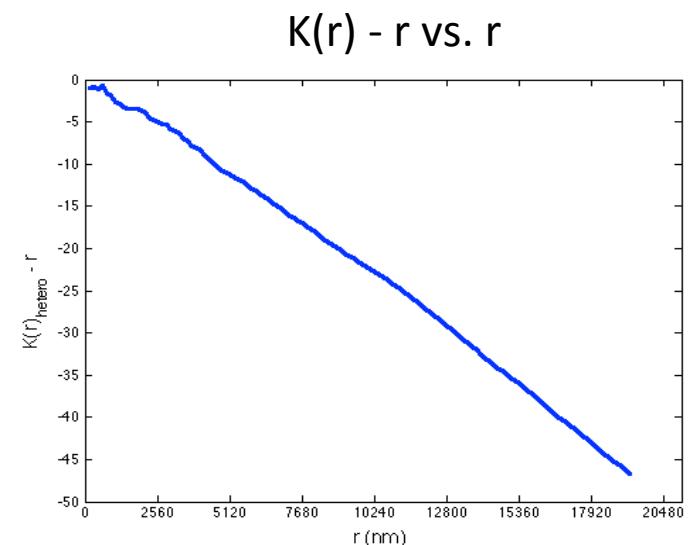
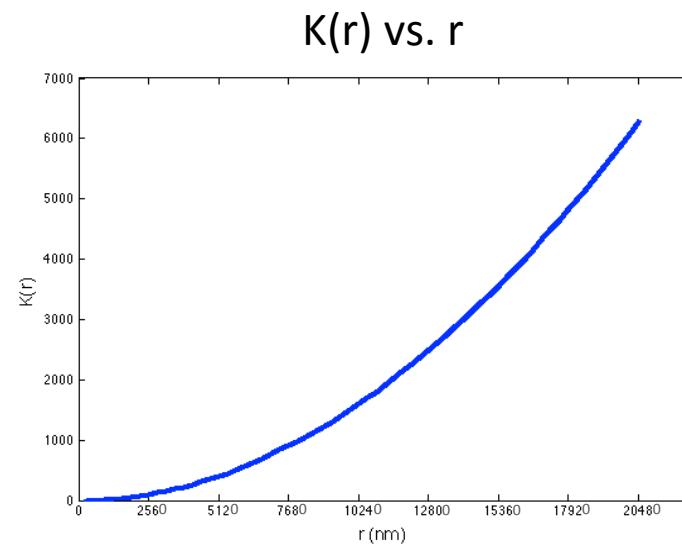
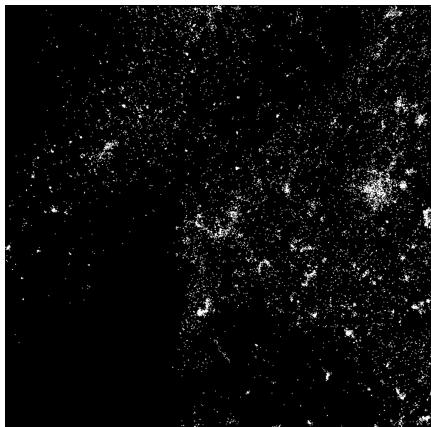


# Ripley's K Function

Homogeneous  
Distribution



Heterogeneous  
Distribution



# Pair Correlation Analysis



- A spatial correlation function – whether “auto” or “cross” – can help us characterize the relationships between points.
- When points are homogeneously distributed:

$$g(r)^{peaks} = g(r)^{stoch} + 1$$

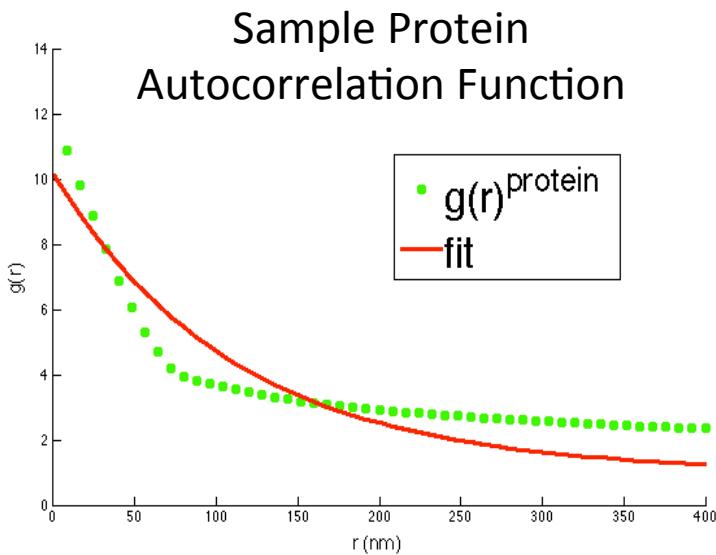
- When points are heterogeneously distributed:

$$\begin{aligned} g(r)^{peaks} &= g(r)^{stoch} + g(r)^{protein} * g(r)^{PSF} \\ &= g(r)^{stoch} + \left( \frac{N}{\psi_{cluster}} e^{(-r/\xi)} + 1 \right) * g(r)^{PSF} \end{aligned}$$

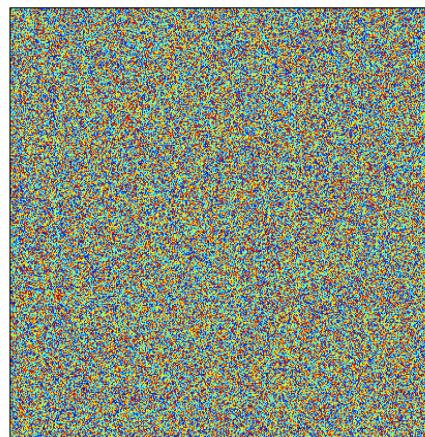
- Running a regression allows us to estimate the radius of a typical cluster, as well as the number and density of proteins in a cluster.
- Performing pair-correlation analysis on a monochromatic data allows us to characterize the clustering behavior of individual proteins; performing this analysis across protein types allows us to characterize colocalization behavior.



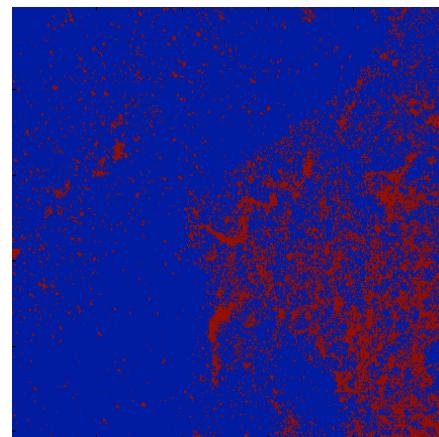
# Pair Correlation Analysis



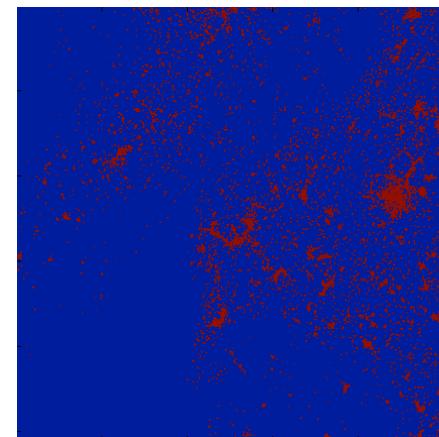
Sample	Estimated Cluster Radius $\xi$ (nm)	Estimated Cluster Population
Homogeneous	0.84	0.11
Clathrin Light Chain	469.58	2131.9
Transferrin Receptor	111.42	201.16
Dynamin	135.88	339.40



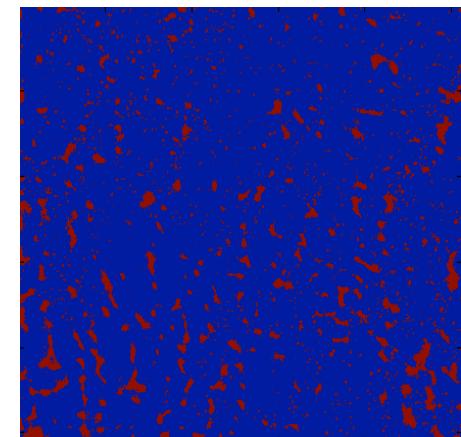
Homogeneous  
Distribution



Clathrin Light Chain



Transferrin Receptor



Dynamin



# Pair Correlation Analysis



- We need to modify our model to include:
  - Stochastic autocorrelation function, which corrects for overcounting
  - PSF autocorrelation function, which accounts for localization error
- Cross-correlation functions should also be calculated.
- Current  $\xi$  and  $N$  estimates are too dependent on initial inputs; more sophisticated parameterization methods are available.
- Algorithm refinement is currently in progress!

**Preliminary Conclusion:** Clustering and colocalization were observed and tentatively quantified.



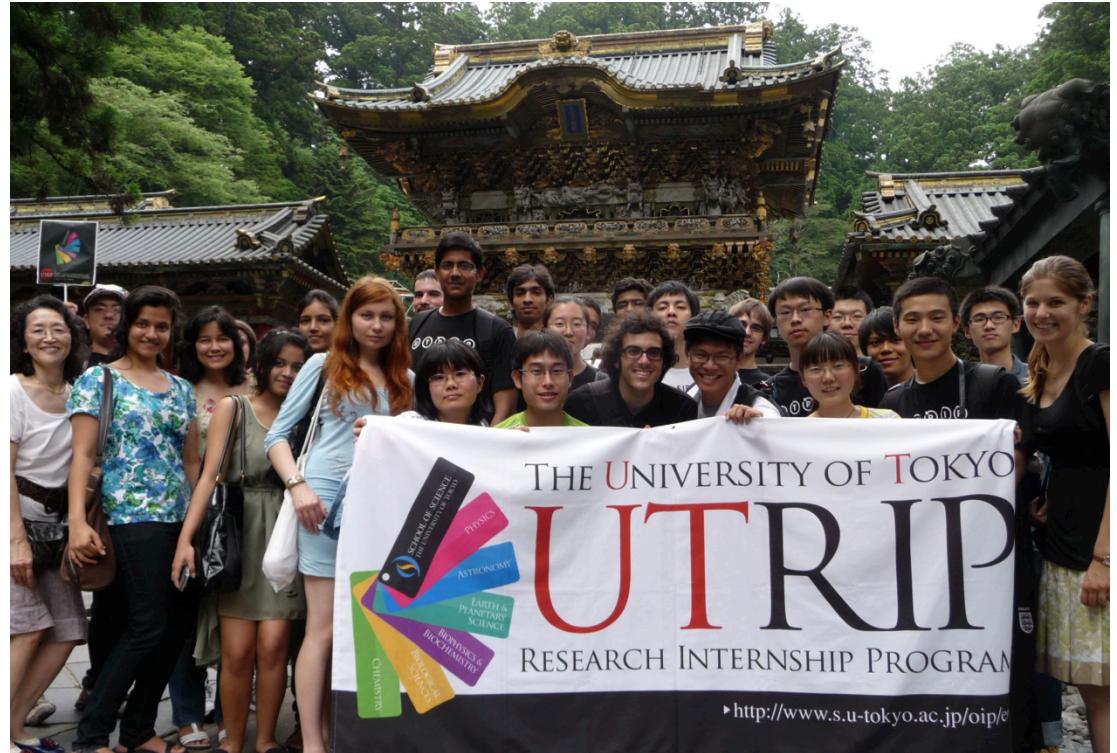
# Summary

- Super-resolution imaging through photoactivated localization microscopy provides a powerful means for circumventing the diffraction limit.
- Antibody-conjugation and chimeric protein expression were used to fluorescently label transferin receptor, clathrin light chain, and dynamin - three proteins thought to collectively mediate endocytosis.
- The fluorescent probes were imaged with confocal and TIRF microscopes using 3 distinct laser lines.
- Cursory data analysis corroborated the clustering and colocalization hypothesis; a more rigorous quantitative treatment using Ripley's K-function and Pair Correlation is currently underway.



# Acknowledgements

- Professor Ozawa
- Yusuke Nasu
- Ozawa Lab
- Mrs. Soeda
- Friends of Todai
- All of you!



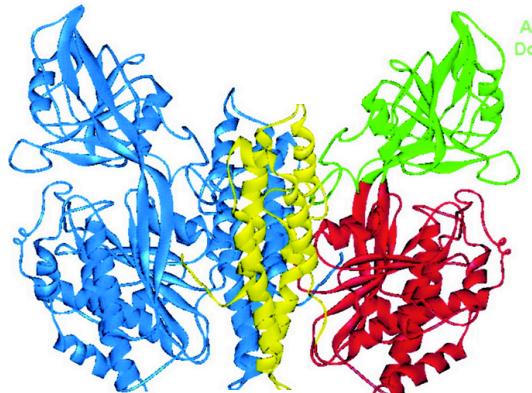
ご静聴ありがとうございました！



# **SUPPLEMENTARY INFORMATION**

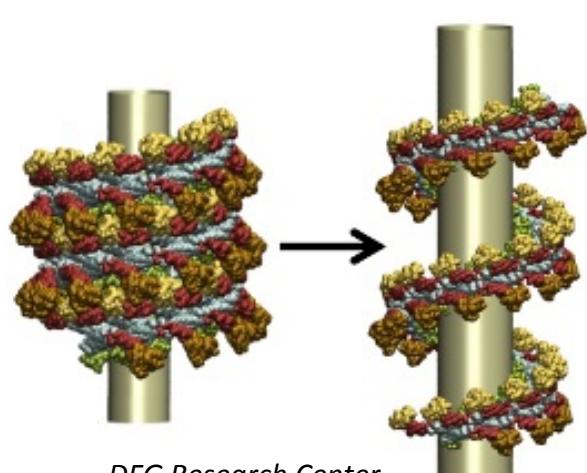


# Target Molecules

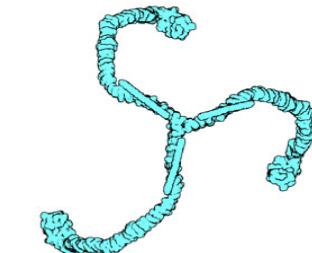
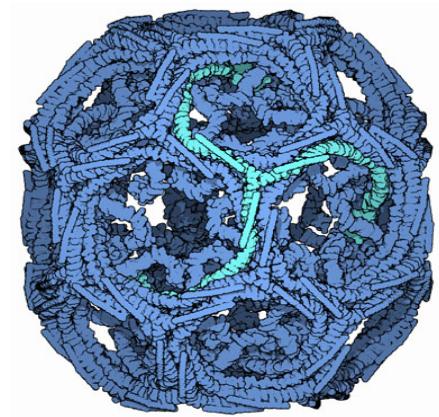


*Pharmacological Reviews,*  
54 (4), 2002

**Transferrin Receptor:** a transmembrane protein that selectively binds to an iron-bearing transferrin molecule



**Dynamin:** a GTPase thought to mediate plasma membrane scission by mechanical twisting

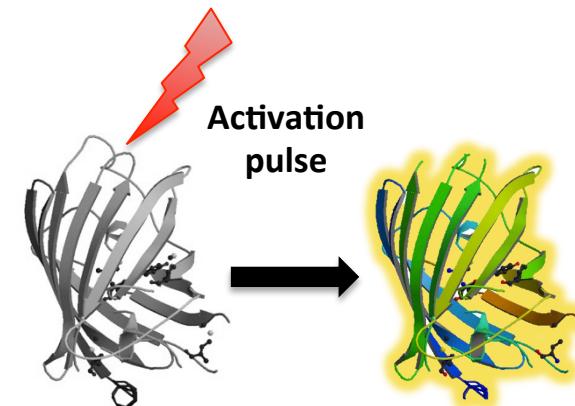
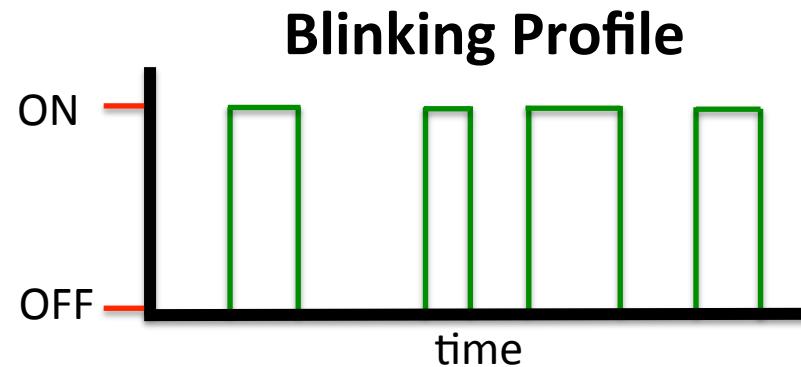


*RCSB PDB Molecule of the Month, April 2007*



# Photoswitching

- A vital criterion for successful PALM imaging is the availability of **photoswitching fluorophores** that can toggle between dark and bright states.
- Fluorescent Intermittency: The probe oscillates randomly between dark and bright states under “continuous excitation.” E.g. AlexaFluor 647 (fluorescent dye)
- Optical Highlighting: Fluorescence is triggered by irradiation with a specific activation wavelength. E.g. PAmCherry (fluorescent protein)



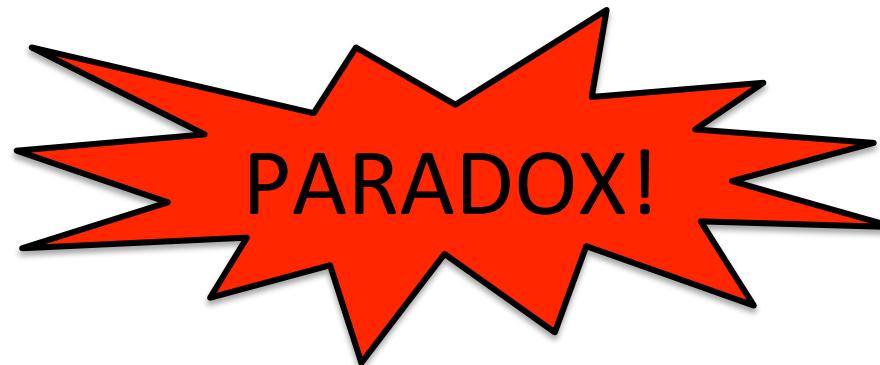
# An Inherent Methodological Trade-Off



- Spatial resolution: optimized when number of emitted photons is maximized. Recall:

$$\Delta_{loc} \approx \frac{\sigma_{PSF}}{\sqrt{N}}$$

- Temporal resolution: optimized when frame rate is maximized.
- HOWEVER, a longer exposure time (lower frame rate) leads to more photons per image (greater N).

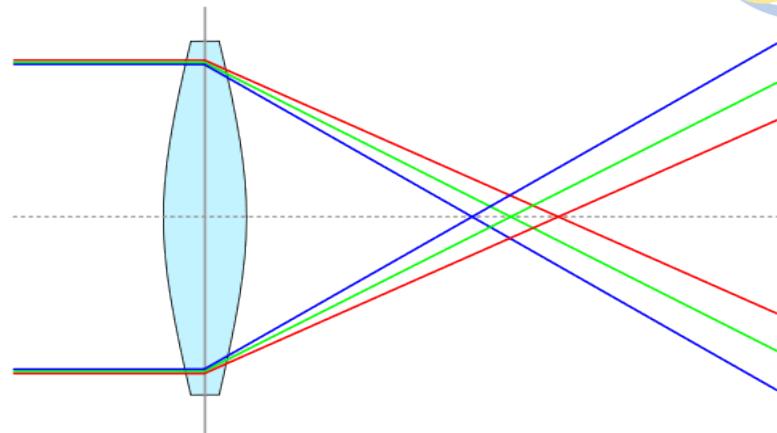
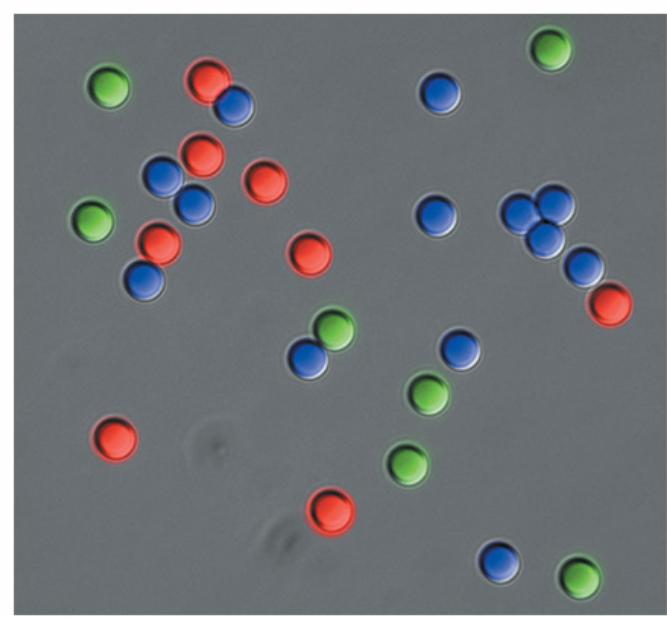


- We ultimately selected an acquisition rate of between 20 and 30  $\mu\text{s}$  per frame (depending on the relative brightness of each fluorescent probe).

# An Additional Obstacle: Chromatic Aberration



- The refractive index is wavelength dependent; therefore, when passing through a lens, each color converges on a slightly different focal point.



- In multicolored fluorescent imaging, this could mean that separate images are nonsuperimposable, thus precluding colocalization analysis.
- This effect can be mitigated by polystyrene microspheres conjugated with fluorescent dyes, which emit light at known wavelengths and can assist in spatial calibration.

# For Further Study: Computational Approaches

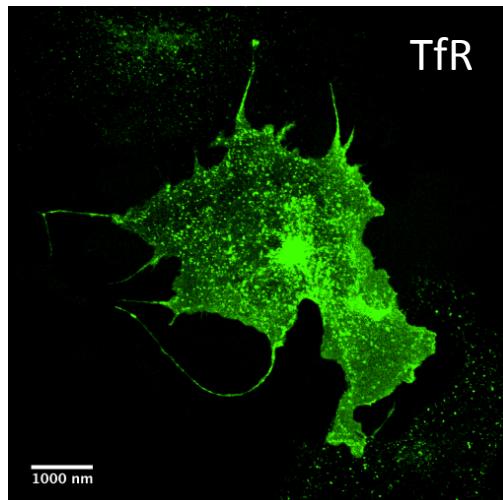


- *in silico* docking simulations are a powerful complement to experimental techniques, offering a high-resolution glimpse into protein-protein interactions.
- HADDOCK biomolecular docking software employs a 3-part algorithm to probe configurational space and estimate the best docking orientation:
  - Rigid body minimization
  - Semi-flexible refinement
  - Fine-tuning in explicit solvent
- We are in the process of performing docking simulations on all-atom models of TfR and dynamin (with PDB crystal structures and CPoRT Prediction Interface results as input).
- Analysis in progress.

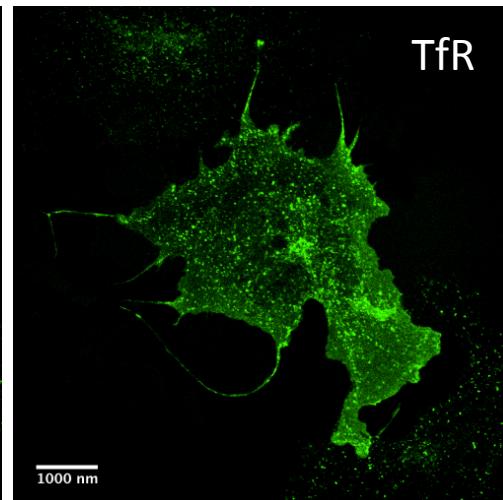
# CONFOCAL IMAGING



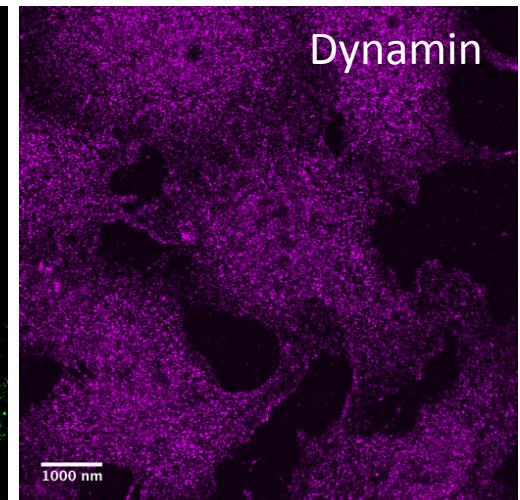
Before PAmCherry  
Photoactivation



After PAmCherry  
Photoactivation



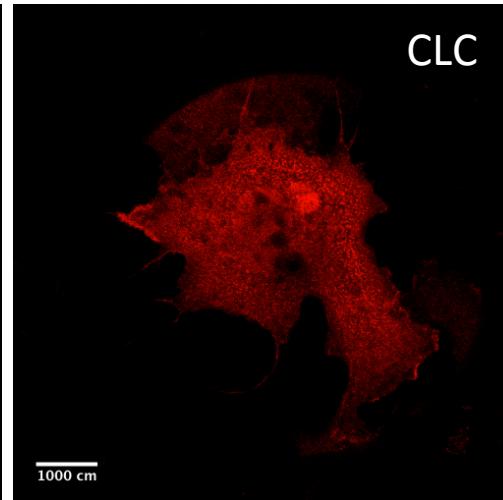
Dynamin



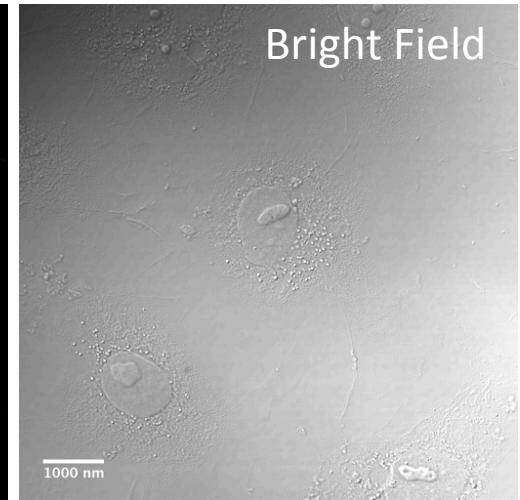
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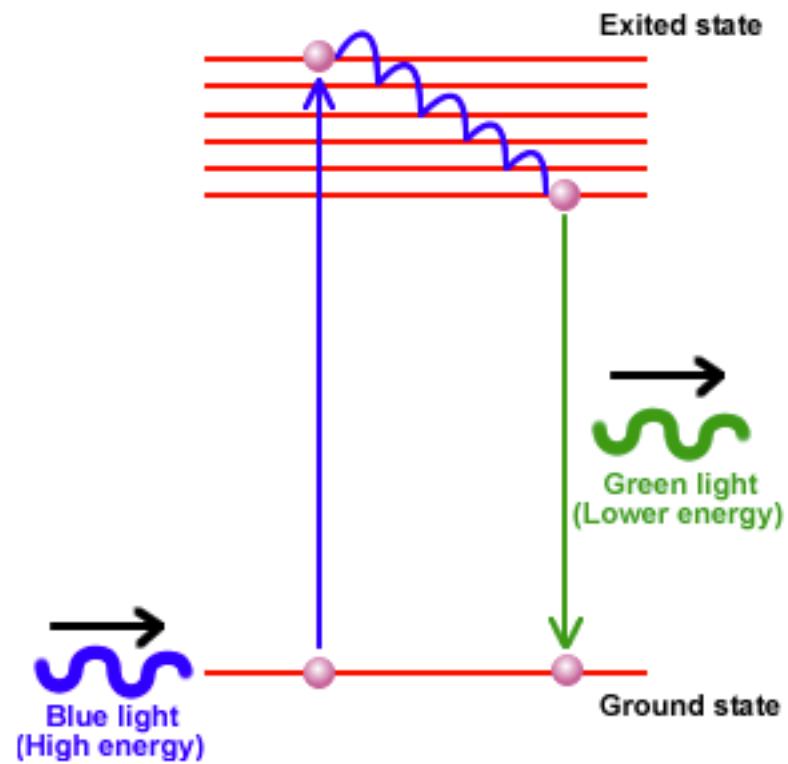


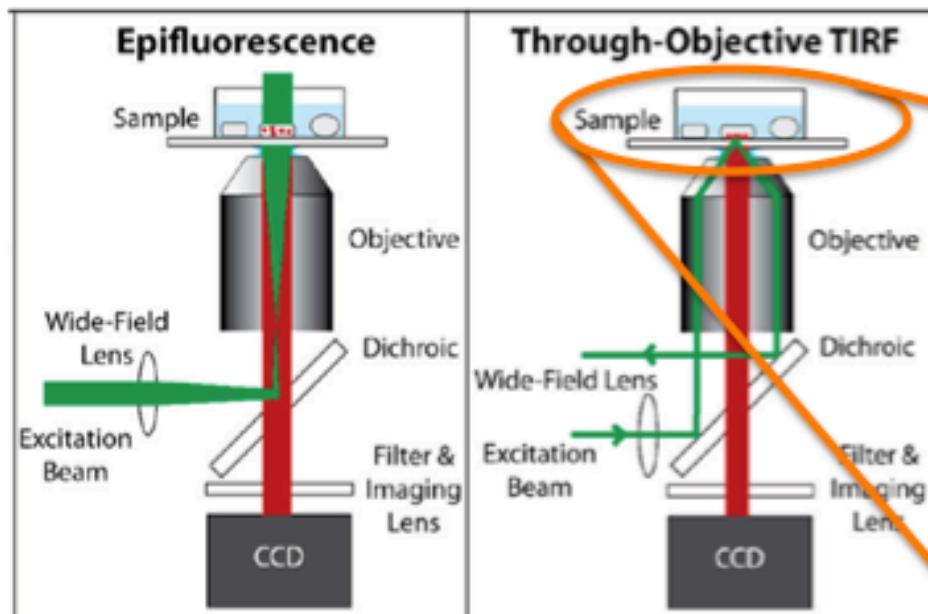
CLC



Bright Field







*Analytical Chemistry, 82 (6), 2010*

