

# Total Internal Reflection Fluorescence Microscope Characterization for Imaging Fluorescence Correlation Spectroscopy

Presenter: Alon Shapiro

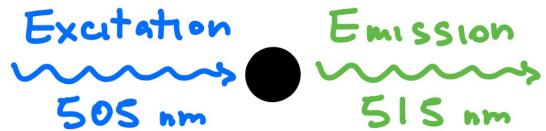
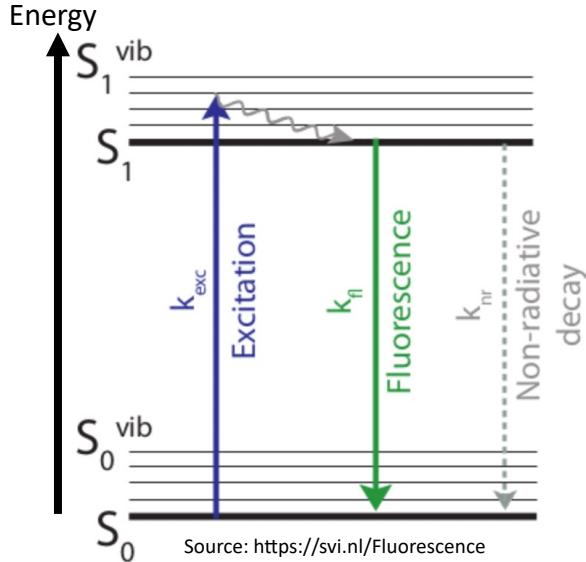
Supervisor: Dr. Paul Wiseman

Co-supervisor: Ahmad Mahmood (PhD candidate)

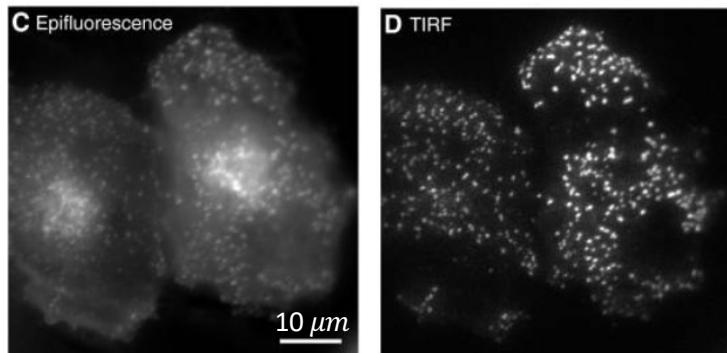
April 7, 2022

# Introduction

Fluorescence: Electron excitation

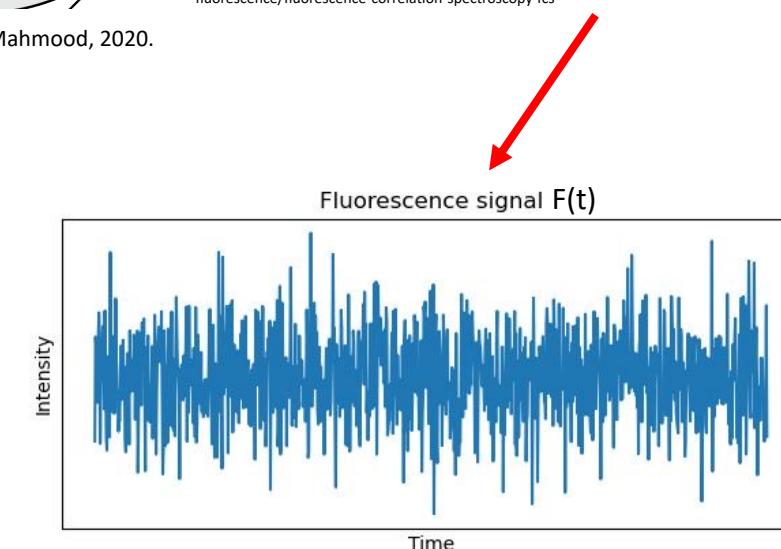
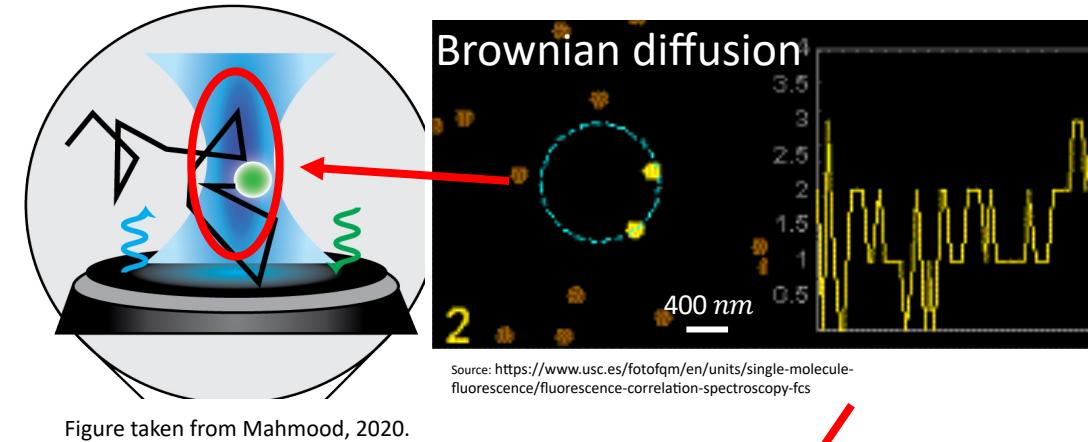


HeLa cells with a GFP-labelled protein



Source: <https://www.photometrics.com/learn/single-molecule-microscopy/tirf-microscopy>

Fluorescence correlation spectroscopy (FCS): fluctuation analysis



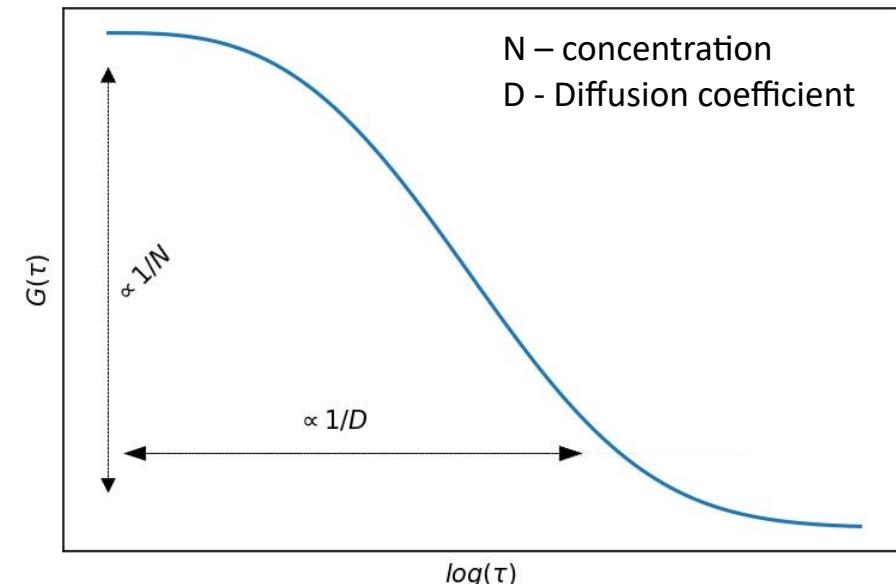
# Introduction

**Autocorrelation function (ACF): measure of self-similarity**

$$G(\tau) = \frac{\langle \delta F(t) \cdot \delta F(t + \tau) \rangle}{\langle F(t) \rangle^2}$$

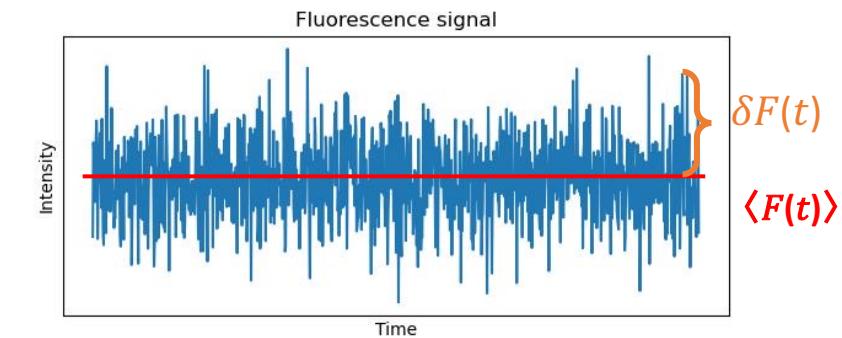
(Elson, 2011)

$F(t)$  : Fluorescence signal over time  
 $\tau$  : Time lag



Fluctuation definition:

$$\delta F(t) = F(t) - \langle F(t) \rangle$$



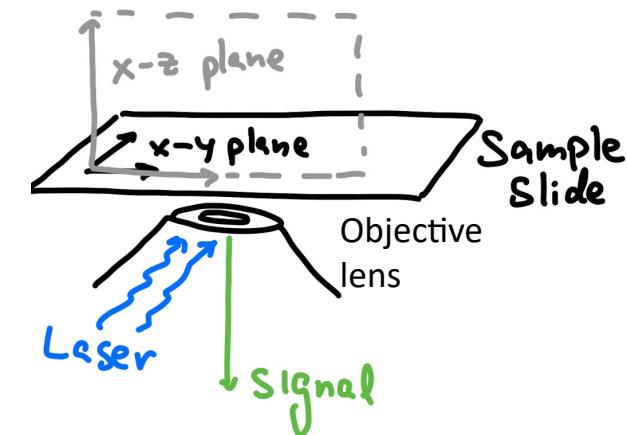
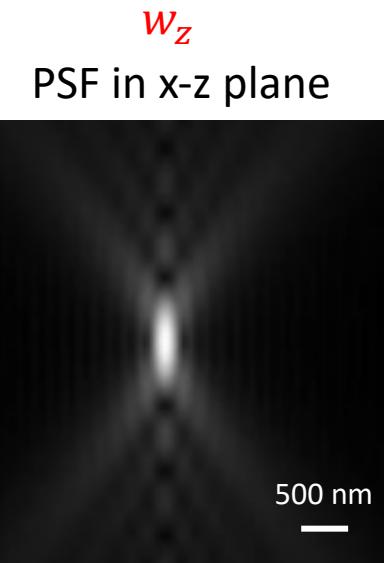
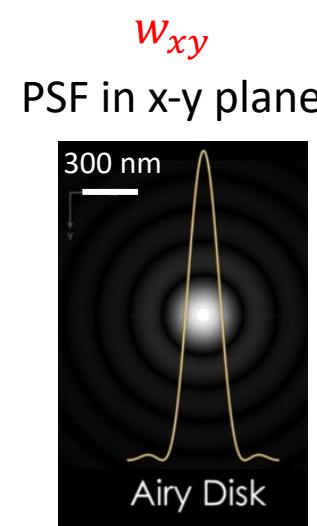
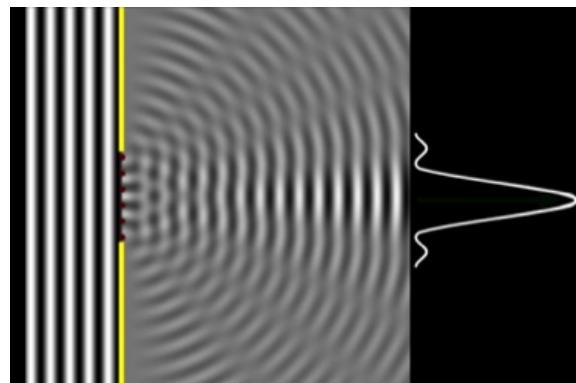
ACF fitting function:

$$g(\tau) = G_{\infty} + \frac{1}{N/A_{\text{eff}}} \cdot \frac{1}{a^2} \cdot \sum_{i=1}^3 \beta_i \cdot \left[ \text{erf} \left( \frac{a}{\sqrt{4D_i \tau + w_{xy}^2}} \right) + \frac{\sqrt{4D_i \tau + w_{xy}^2}}{\sqrt{\pi} \cdot a} \cdot \left[ \exp \left( -\frac{a^2}{4D_i \tau + w_{xy}^2} \right) - 1 \right] \right]^2$$

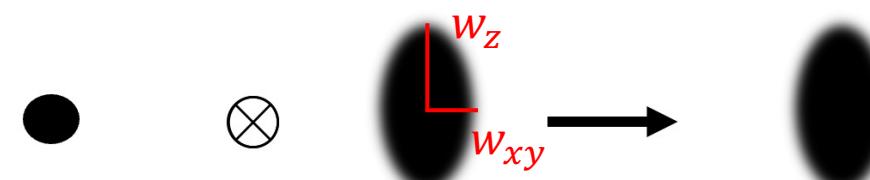
# Introduction

## Point spread function (PSF)

Diffraction of light causes interference as light passes through optical apparatus

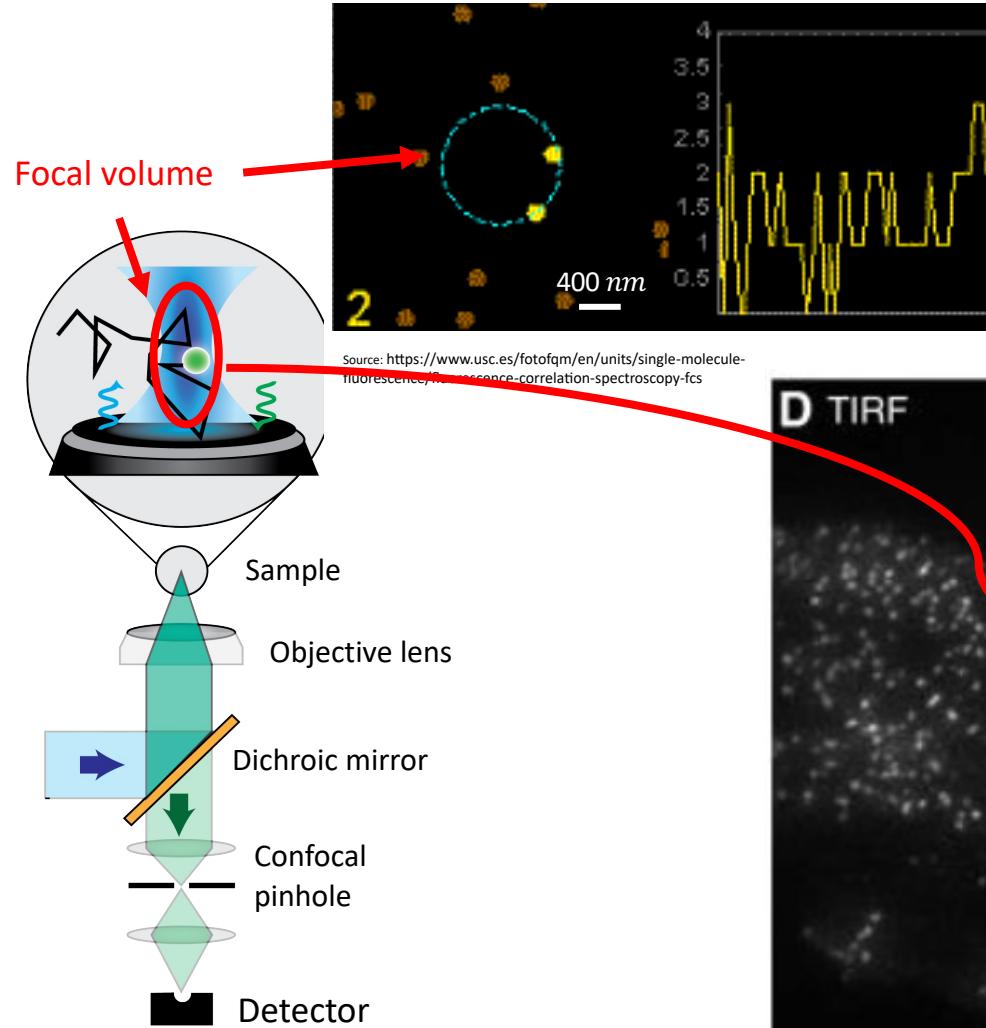


Diffraction-limited resolution



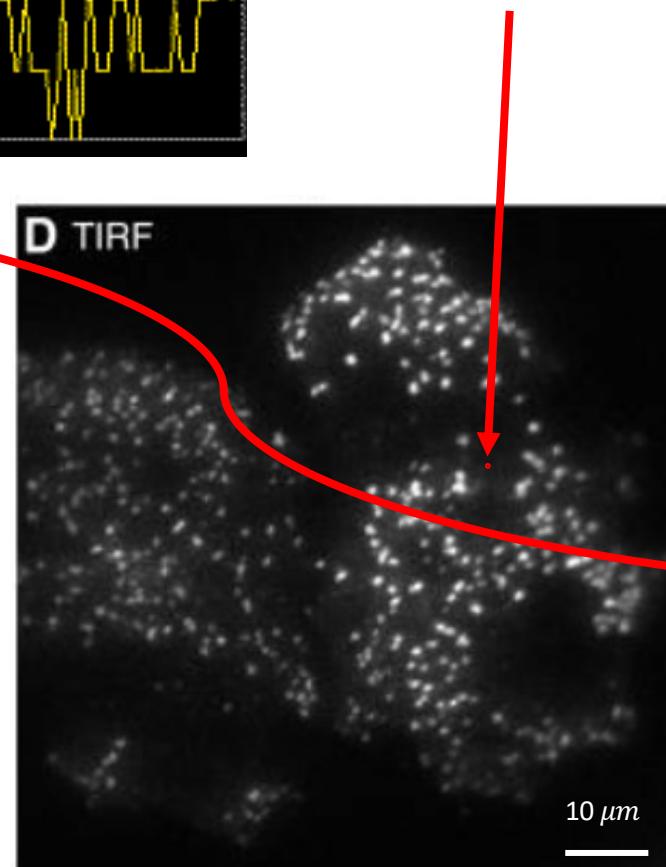
# Introduction

## Confocal FCS



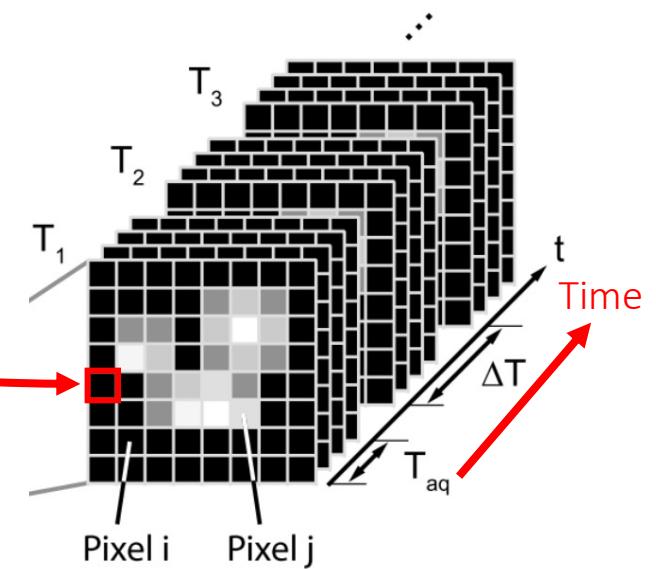
## Biological motivation?

Focal volume size in a TIRF image for comparison



Biological Processes occur on much larger scales

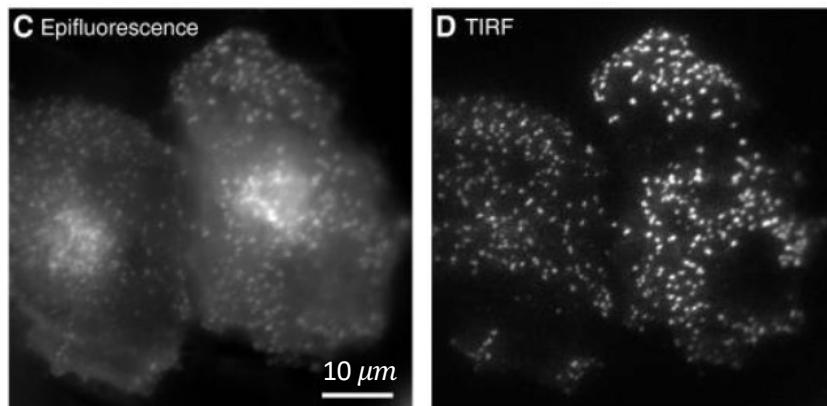
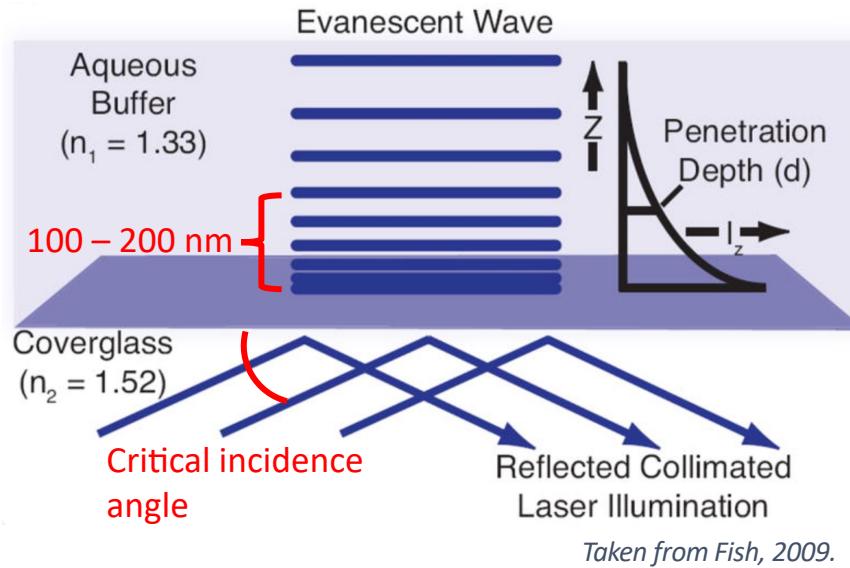
## Imaging FCS (FCS with a rapid camera)



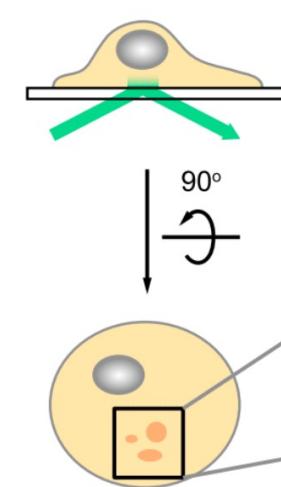
Taken from Kannan et al. 2007.

# Introduction

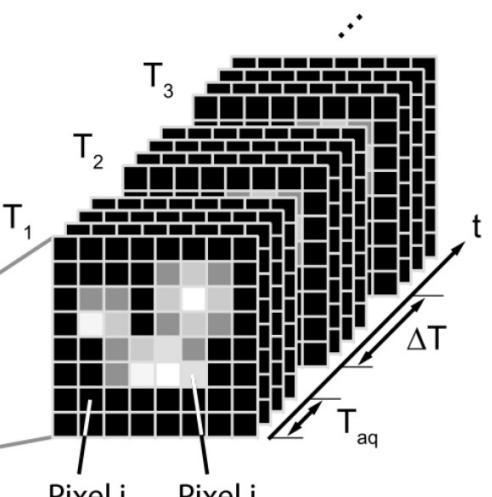
## Total-Internal Reflection Fluorescence (TIRF)



Live cell TIRF imaging



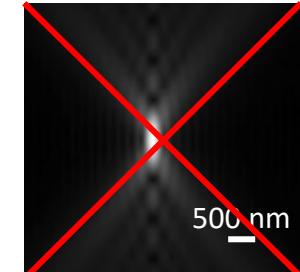
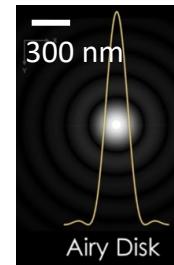
Raw TIRF movies



*Taken from Kannan et al. 2007.*

TIRF is confined to a very thin plane, effectively only has x-y component of PSF:

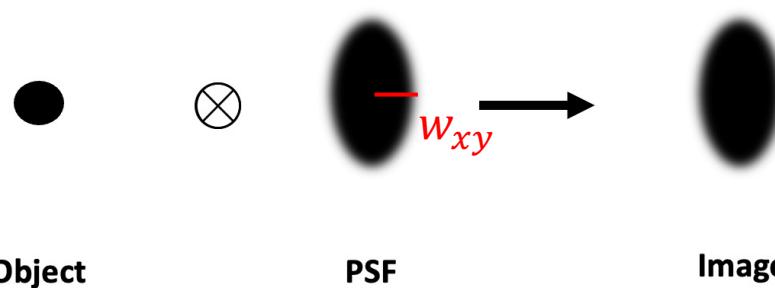
$w_{xy}$



# Goals

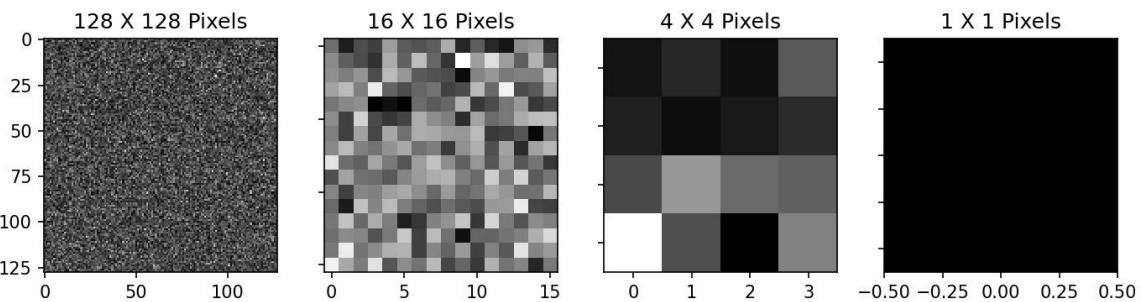
- Calibration (Determination of PSF  $w_{xy}$  parameter)
- Validation against Confocal FCS

Required for ACF fitting!

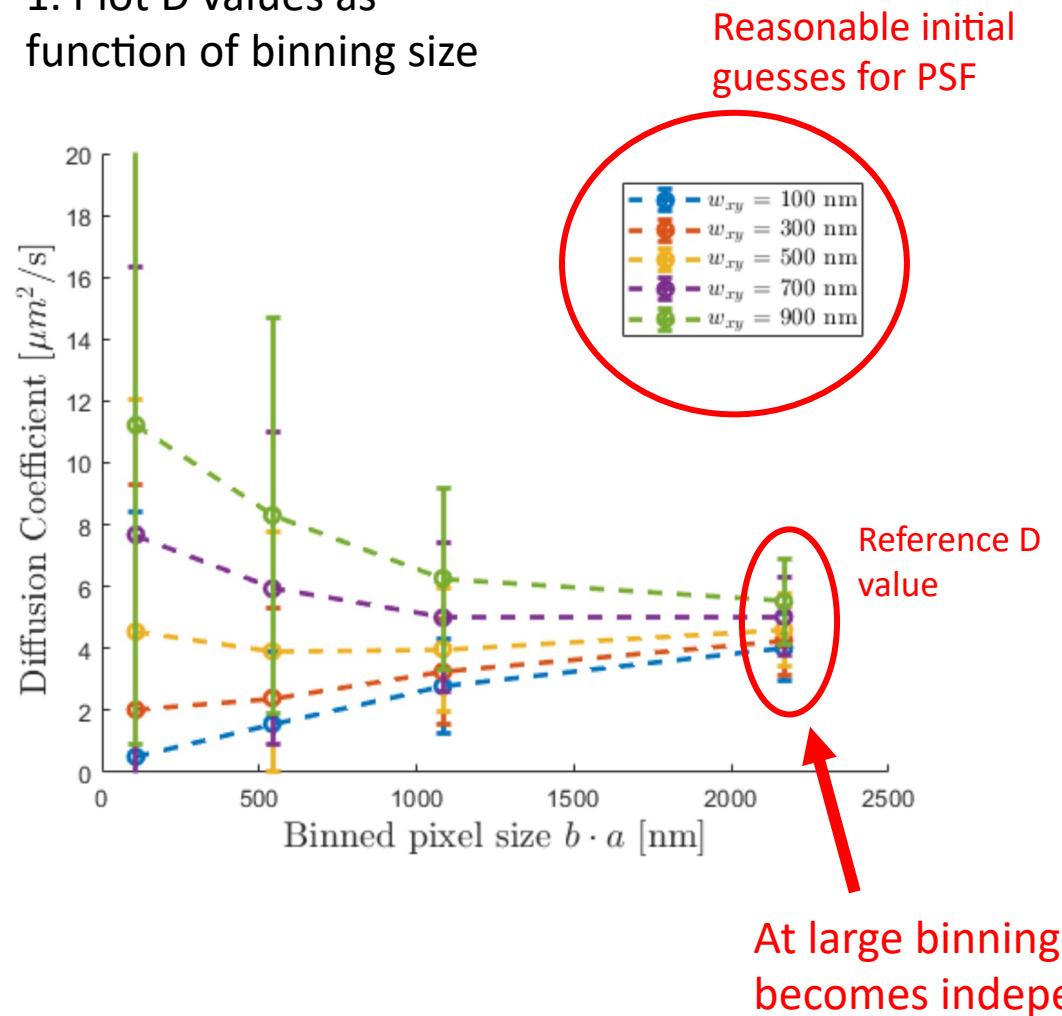
$$g(\tau) = G_\infty + \frac{1}{N/A_{\text{eff}}} \cdot \frac{1}{a^2} \cdot \sum_{i=1}^3 \beta_i \cdot \left[ \text{erf} \left( \frac{a}{\sqrt{4D_i\tau + w_{xy}^2}} \right) + \frac{\sqrt{4D_i\tau + w_{xy}^2}}{\sqrt{\pi} \cdot a} \cdot \left[ \exp \left( -\frac{a^2}{4D_i\tau + w_{xy}^2} \right) - 1 \right]^2 \right]$$


The diagram illustrates the convolution process. It shows an "Object" (a small black circle), followed by a tensor product symbol ( $\otimes$ ), then a "PSF" (a Gaussian-shaped blob with a red horizontal line labeled  $w_{xy}$  indicating its width), and finally an "Image" (a larger, more spread-out blurred version of the object). An arrow points from the PSF to the Image.

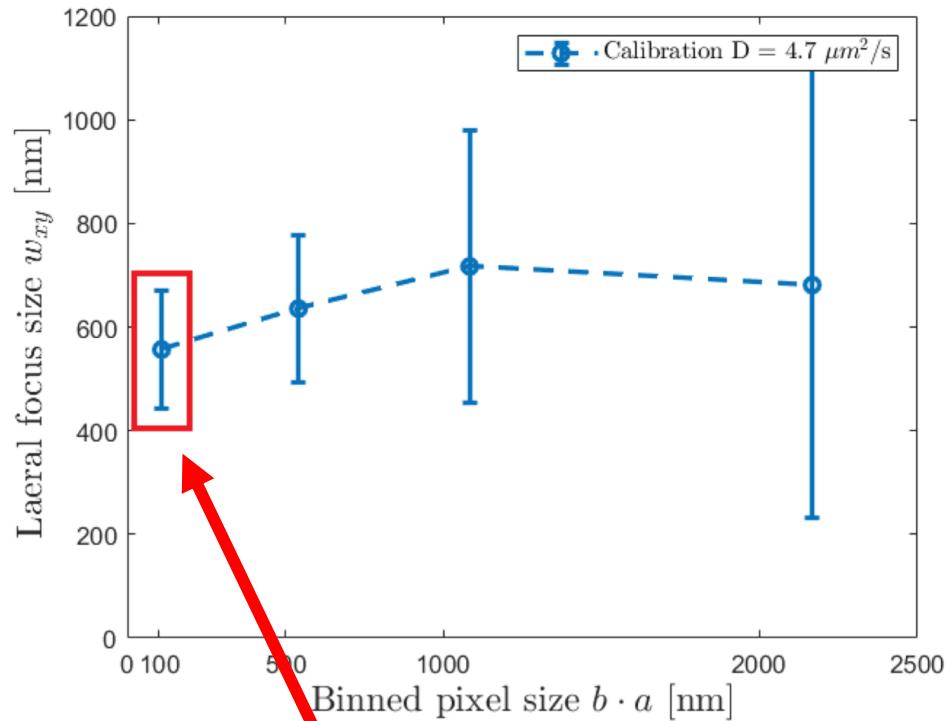
# Goal: Calibration



1. Plot D values as function of binning size



2. Plot PSF as function of binning size, with now known reference D value

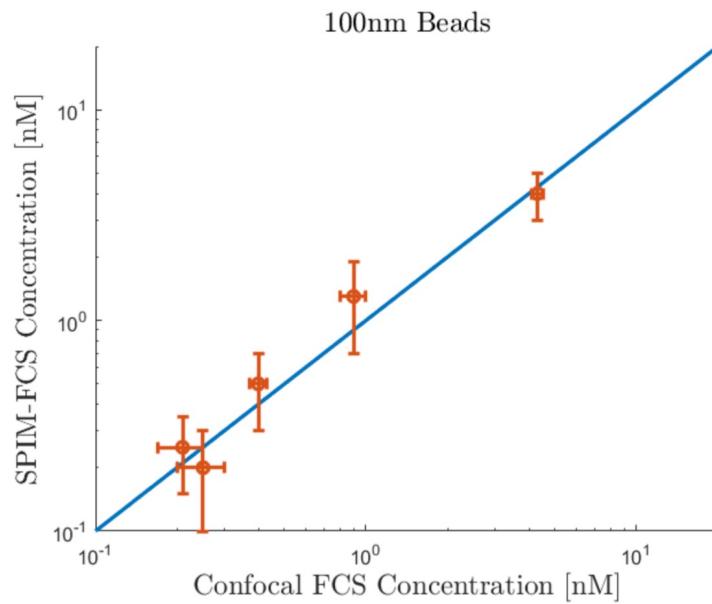


At large binning sizes, ACF becomes independent of PSF

PSF parameter at smallest binning size is the true parameter

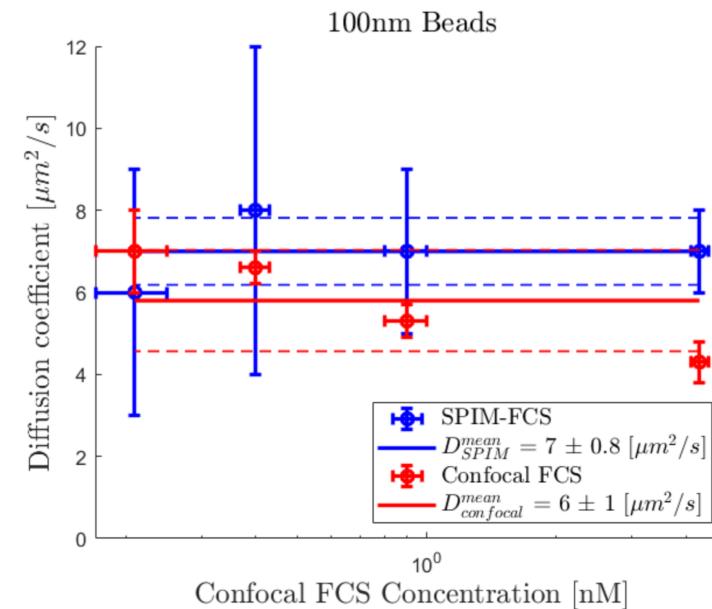
# Goal: Validation against confocal microscope

1. Plot sample concentration measurements from TIRF vs. Confocal FCS



A linear plot will indicate agreement

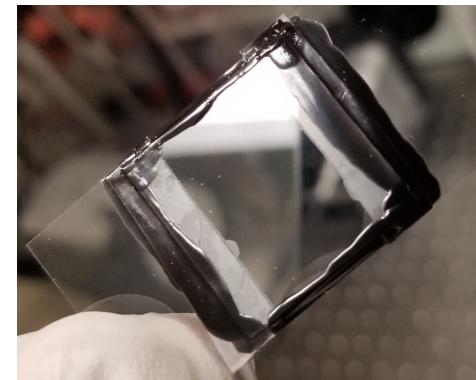
2. Plot D vs. sample concentration measurements for both TIRF and confocal FCS



Measurements from each microscope within error bars of each other will indicate agreement

# Methods

- Sample: 100 nm fluorescent beads diffusing in water at room temperature
- Diffusion coefficient should be around  $4.5 \pm 1 \mu\text{m}^2/\text{s}$  (Stokes-Einstein)

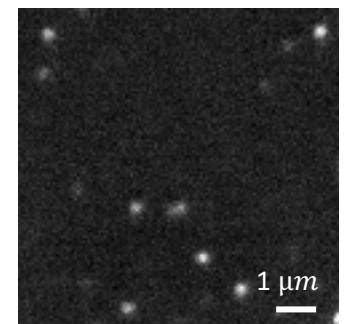
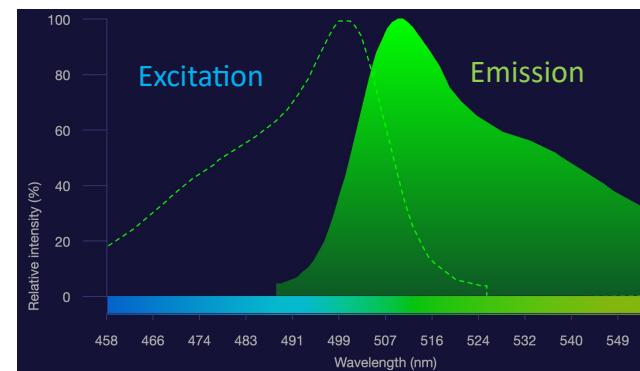


$$D = \frac{k_{\text{B}} T}{6\pi \eta r}$$

*D* - diffusion coefficient  
*r* - spherical particle radius  
*η* - viscosity

- After initial imaging, needed to improve **temporal resolution** and reduce **file size**, analysis software (Quick Fit 3.0) only accepts up to 2 GB files

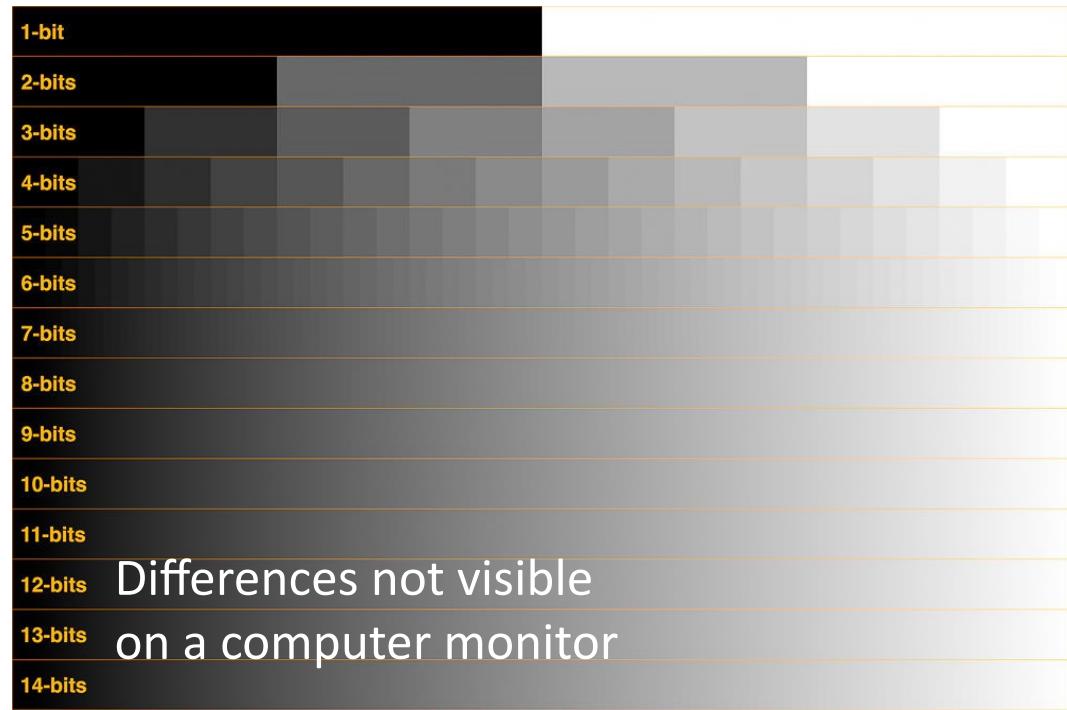
- Constraints:
  - <2 GB file
  - ~150-200K frames for sufficient statistics
  - Improve temporal resolution
- Try 12-bit acquisition (instead of 16)



Should improve frame rate & decrease file size

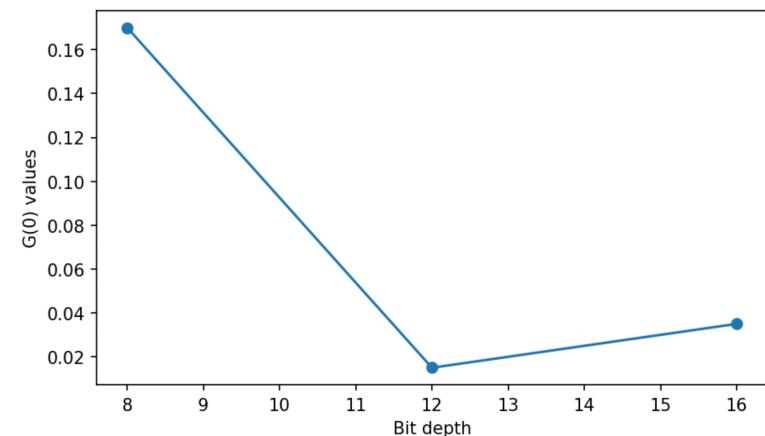
# Methods

- What are bits?
- 1 bit: 2 values, 0 or 1.
- 8 bits: 256 values
- 12 bits: 4096
- 16 bits: 65536
- Bit depth = Size of difference between shades
- Lower bit depth causes loss of information
- Same file size since 12 bits are stored as 16 bits (no 12-bit format)
- Concentration measurement  $G(0)$  different for 16 vs 12 vs 8 bits – fluorescent signal altered, not good



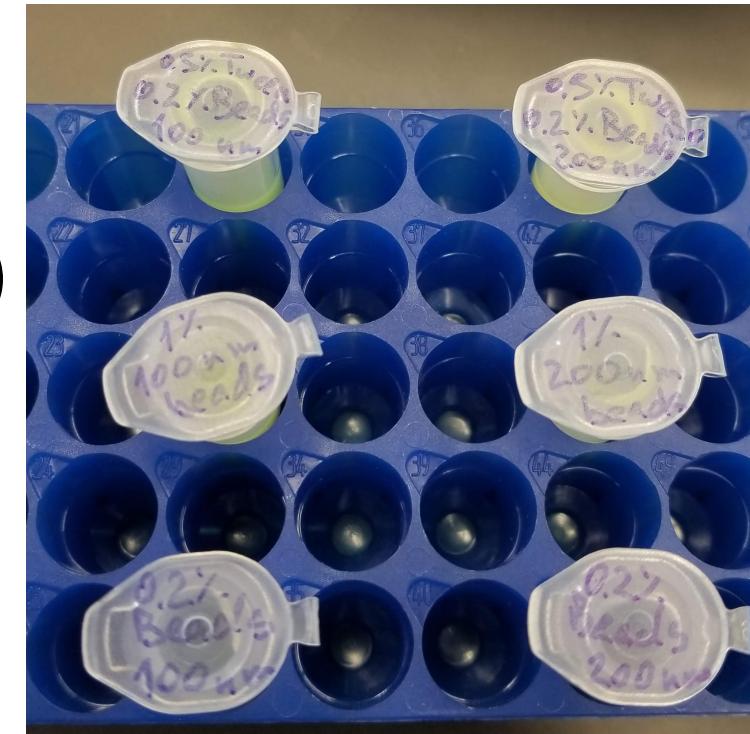
Source: <https://petapixel.com/2018/09/19/8-12-14-vs-16-bit-depth-what-do-you-really-need/>

16-bit 60k frames: ~1.8 GB  
12-bit 60k frames: ~1.8 GB  
8-bit 120k frames: ~1.7 GB



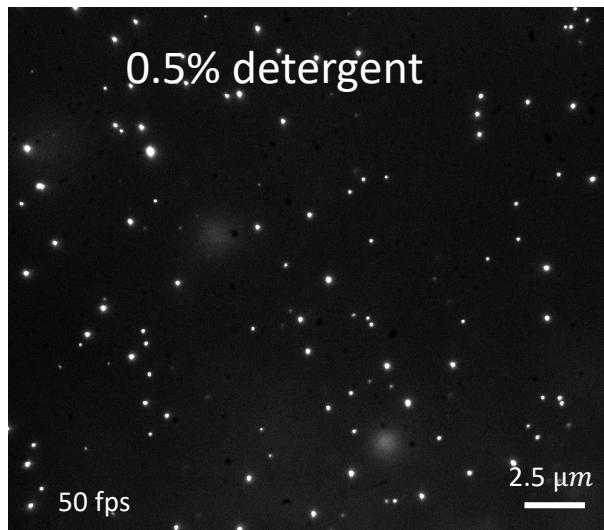
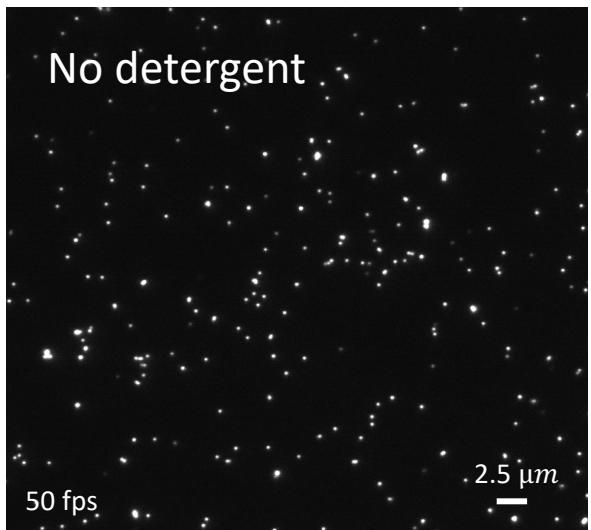
# Methods

- **Reduce diffusion speeds** to make up for temporal resolution
- **Bead immobilization** on glass slide, interferes with imaging
- To solve both these concerns: use detergent (Tween20)
  - Prevents beads immobilization
  - Due to its viscosity, it would slow down diffusion

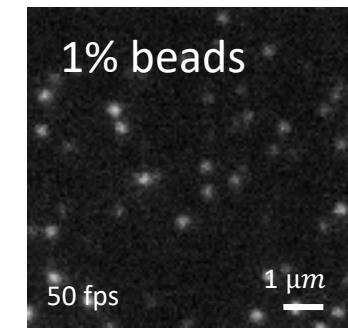
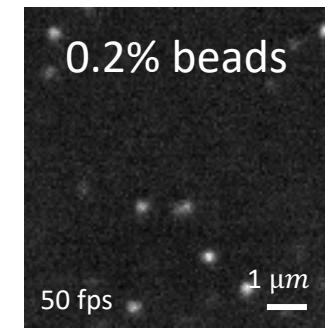
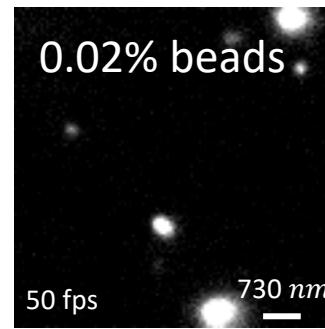


# Methods

Detergent reduces immobilization:

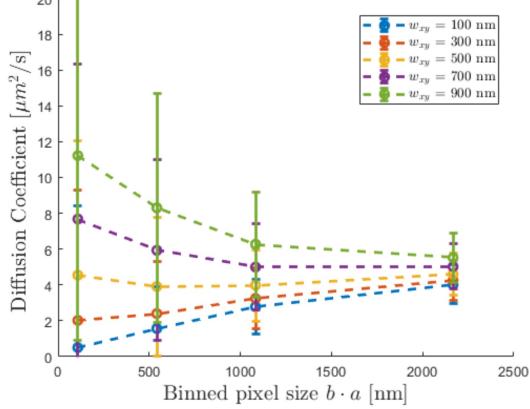


Improve fluorescence signal by making a **more concentrated** sample:

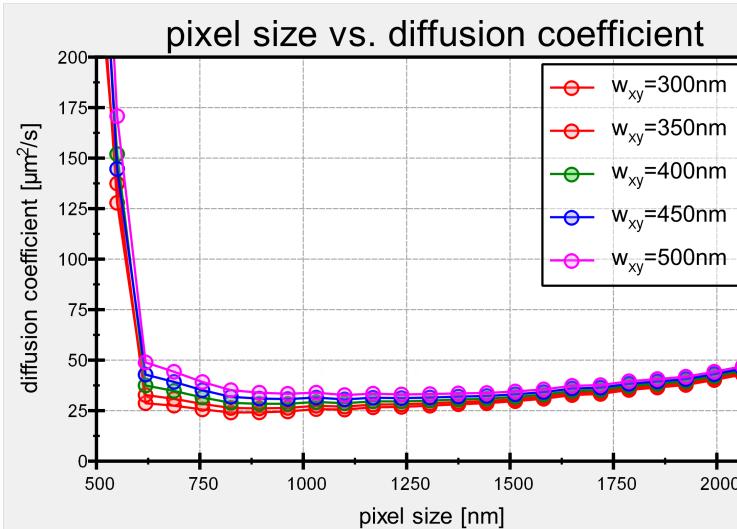


# Results of calibration

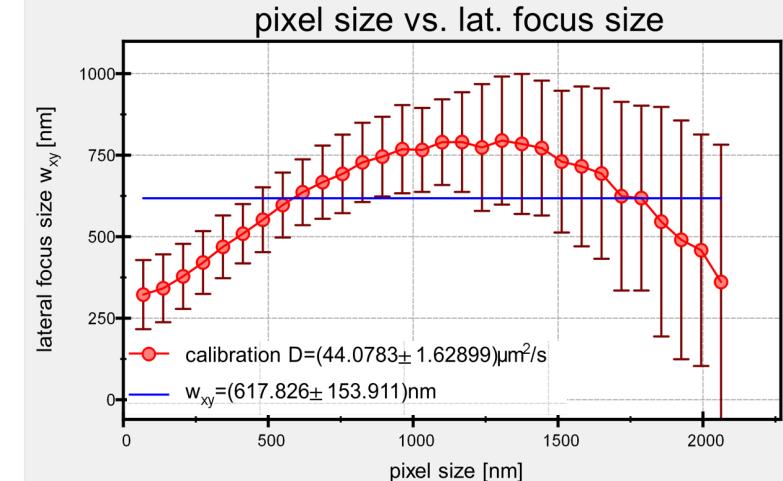
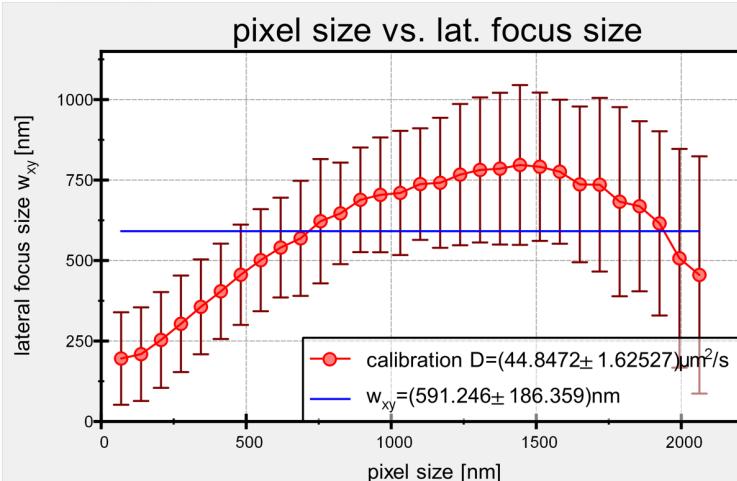
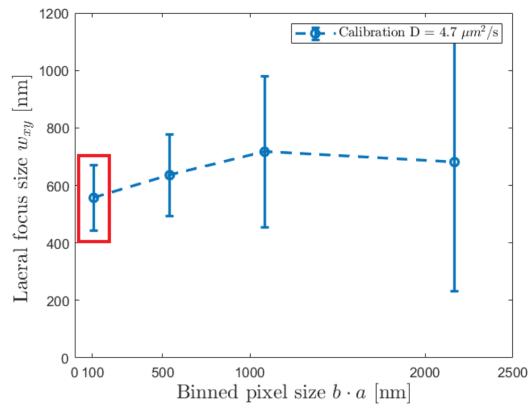
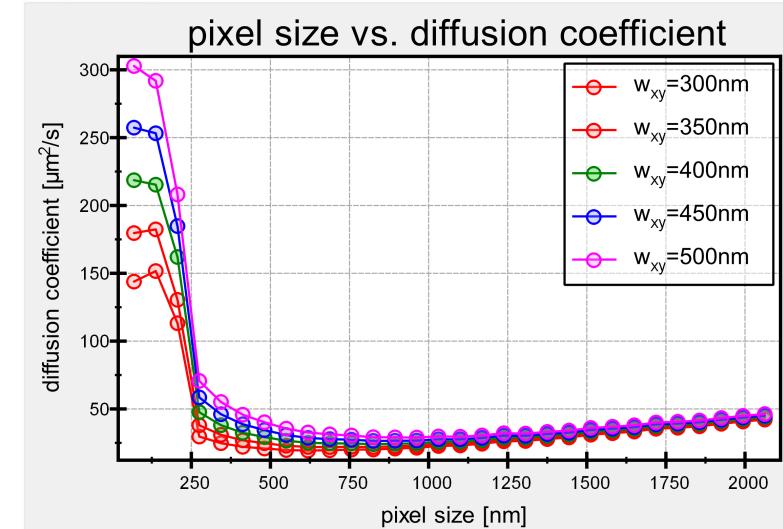
Expected outcome:



0.2 % Beads, 100 nm, 60k frames

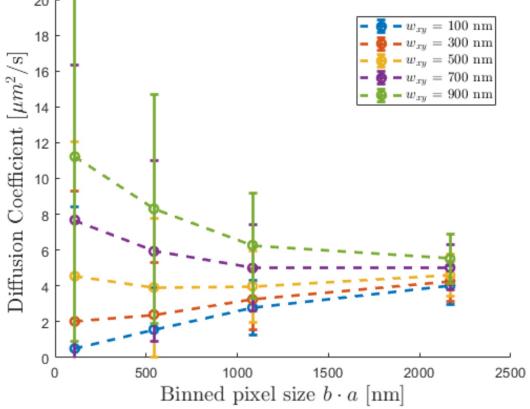


1 % Beads, 100 nm, 60k frames

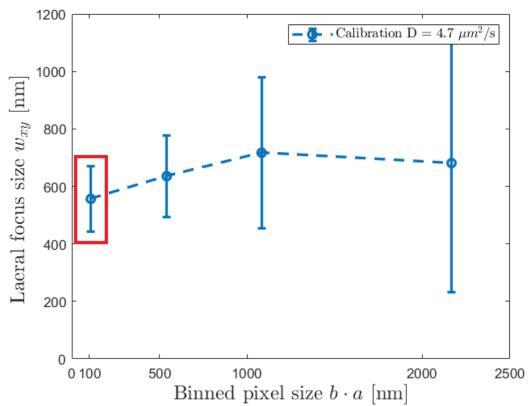


# Interpretation

Expected outcome:



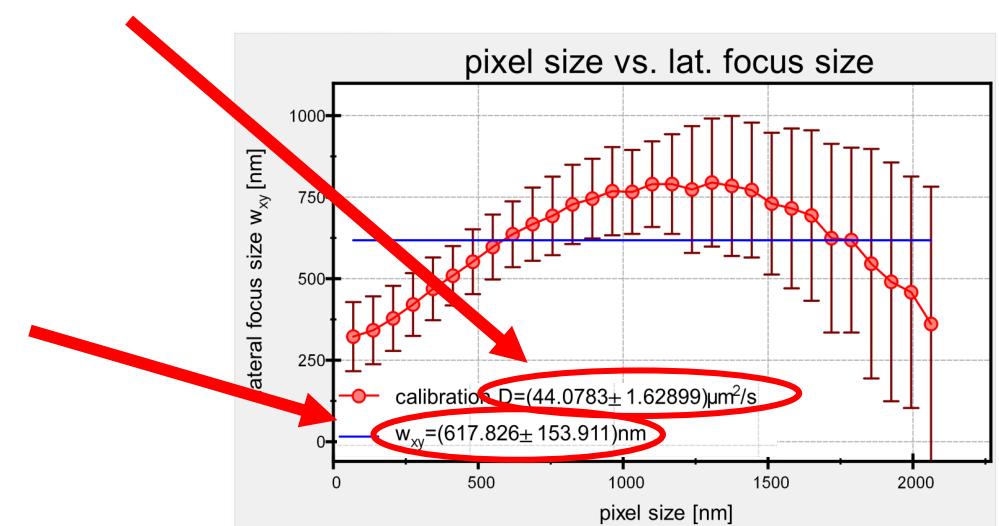
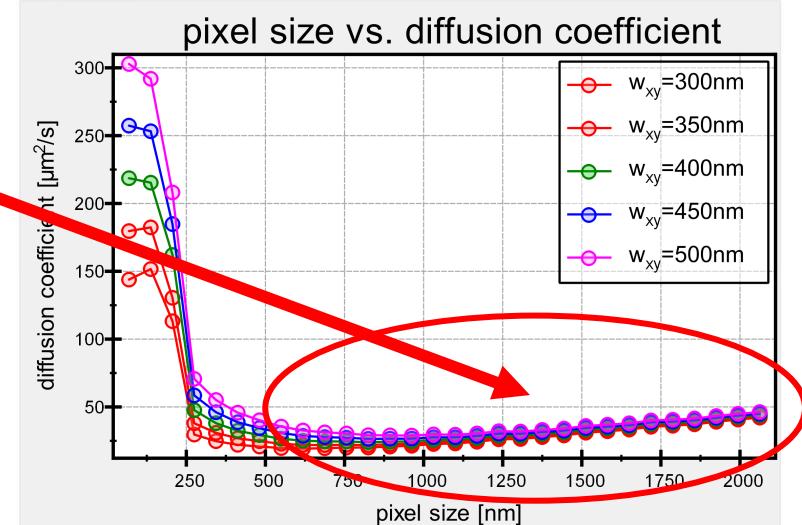
- Upward trend should not be present  
(Diffusion coefficients should be independent of choice of binning)



- Diffusion coefficient values are too high  
(should be  $\sim 4.5 \pm 1 \mu\text{m}^2/\text{s}$ )

- PSF parameter value is too high  
(should be  $\sim 300 \pm 100 \text{ nm}$ )

1 % Beads, 100 nm, 60k frames



# Conclusions and next steps

We're missing something!



Things to try next:

- Try using alternative analysis software (Fiji ImFCS plugin)
- CS / Soft. Eng. Project to look into analysis software more closely (source code available on GitHub)
- Look at bit depth effect on fluorescence signal

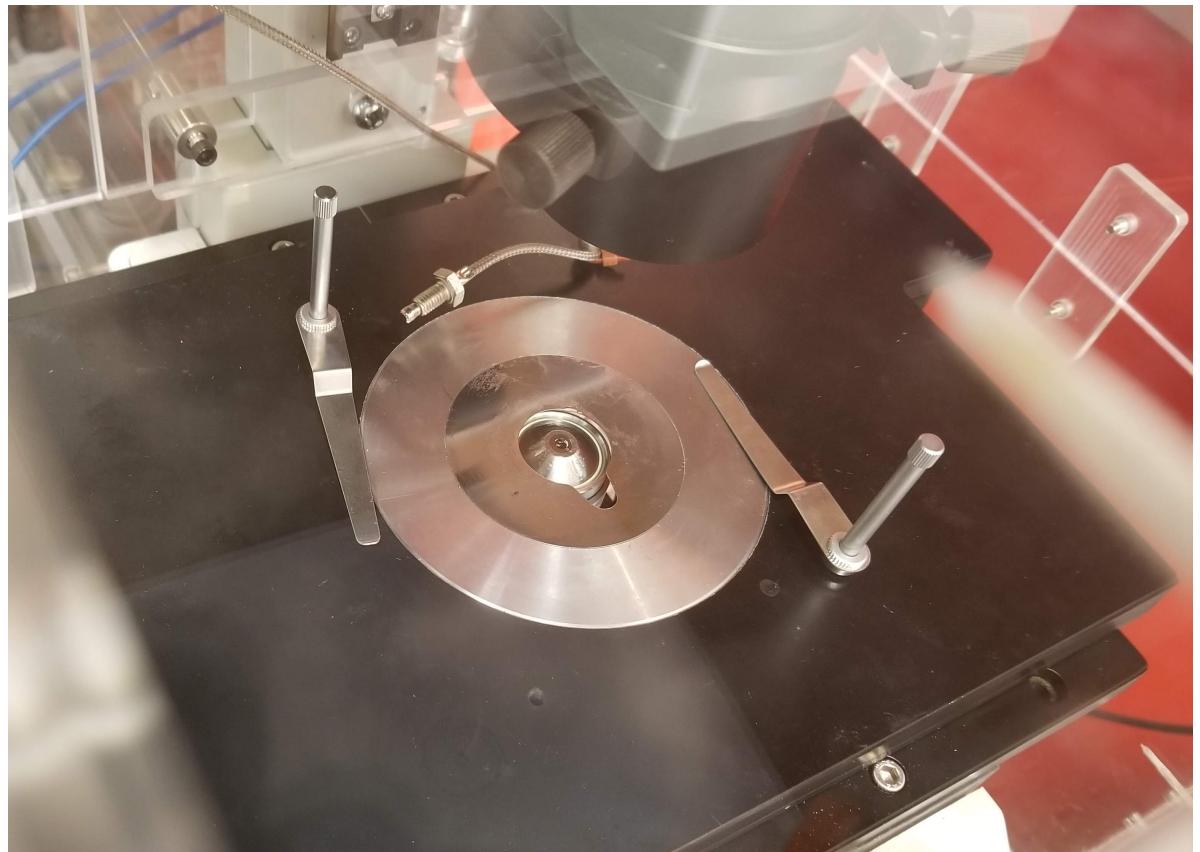
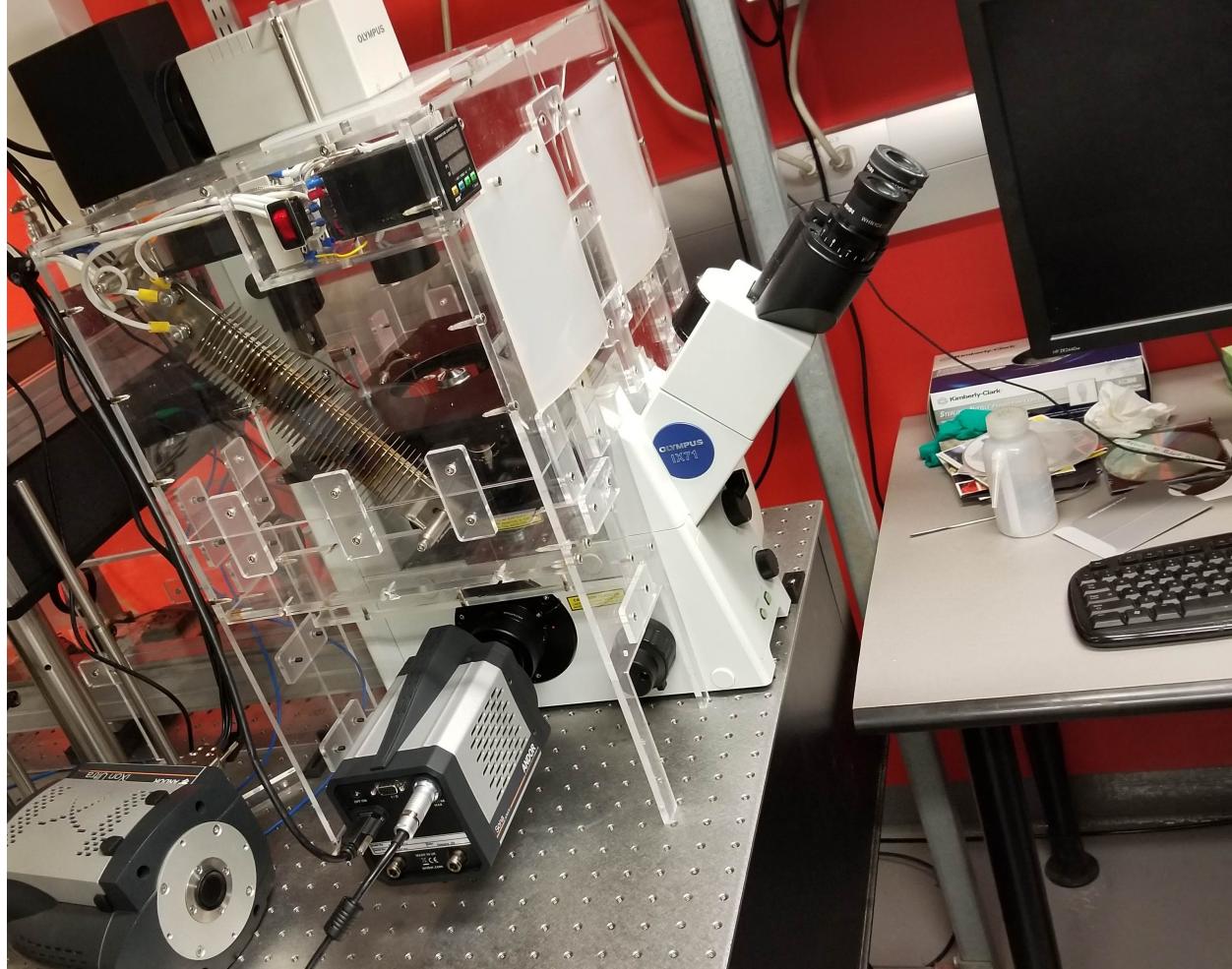
# Acknowledgement

I want to thank Prof. Paul Wiseman and Ahmad Mahmood (PhD candidate), and everyone in the Wiseman lab for the help they offered me during this project!

# References

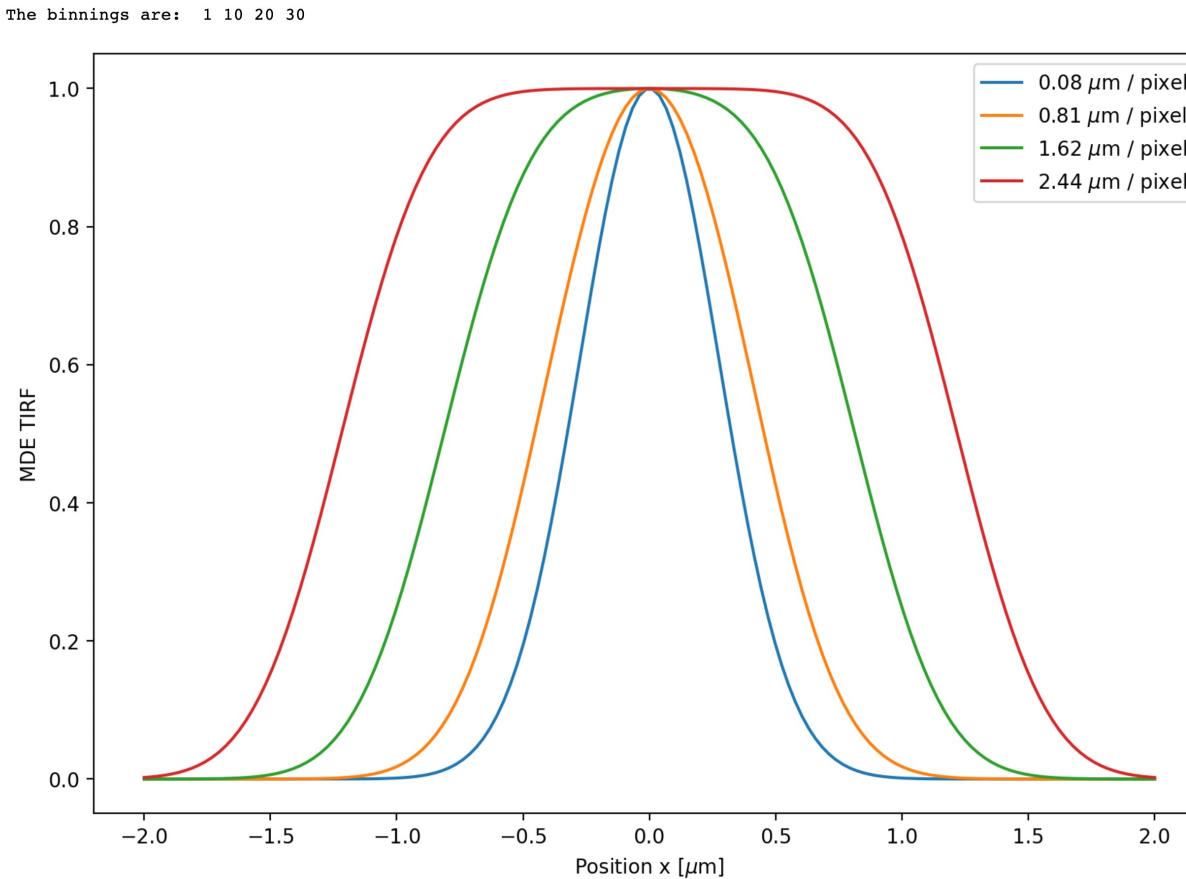
- Milon, Séverine & Hovius, Ruud & Vogel, H & Wohland, Thorsten. (2003). Factors influencing fluorescence correlation spectroscopy measurements on membranes: Simulations and experiments. *Chemical Physics*. 288. 171–186. 10.1016/S0301-0104(03)00018-1
- Ng, X. W., Bag, N., & Wohland, T. (2015). Characterization of Lipid and Cell Membrane Organization by the Fluorescence Correlation Spectroscopy Diffusion Law. *Chimia*, 69(3), 112– 119. <https://doi.org/10.2533/chimia.2015.112>
- Mahmood, M.A. (2020). Characterizing a single plane illumination microscope for imagin fluorescence correlation spectroscopy. McMaster University – Department of Physics and Astronomy. URI: <http://hdl.handle.net/11375/26128>
- Sankaran, J., Bag, N., Kraut, R. S., & Wohland, T. (2013). Accuracy and precision in camera-based fluorescence correlation spectroscopy measurements. *Analytical chemistry*, 85(8), 3948–3954. <https://doi.org/10.1021/ac303485t>
- Guo, S. M., Bag, N., Mishra, A., Wohland, T., & Bathe, M. (2014). Bayesian total internal reflection fluorescence correlation spectroscopy reveals hIAPP-induced plasma membrane domain organization in live cells. *Biophysical journal*, 106(1), 190–200. <https://doi.org/10.1016/j.bpj.2013.11.4458>
- Bag, N., Sankaran, J., Paul, A., Kraut, R.S. and Wohland, T. (2012), Calibration and Limits of Camera-Based Fluorescence Correlation Spectroscopy: A Supported Lipid Bilayer Study. *ChemPhysChem*, 13: 2784-2794. <https://doi.org/10.1002/cphc.201200032>
- Kannan, B.S., Guo, L., Sudhaharan, T., Ahmed, S., Maruyama, I., & Wohland, T. (2007). Spatially resolved total internal reflection fluorescence correlation microscopy using an electron multiplying charge-coupled device camera. *Analytical chemistry*, 79 12, 4463-70
- Ross, S. T., Schwartz, S., Fellers, T. J., & Davidson, M. W. (n.d.). Total internal reflection fluorescence (TIRF) microscopy. Nikon's MicroscopyU. Retrieved November 18, 2021, from <https://www.microscopyu.com/techniques/fluorescence/total-internal-reflection-fluorescence-tirf-microscopy>
- Elson EL. Fluorescence correlation spectroscopy: past, present, future. *Biophys J*. 2011;101(12):2855-2870. doi:10.1016/j.bpj.2011.11.012
- Fish KN. Total internal reflection fluorescence (TIRF) microscopy. *Curr Protoc Cytom*. 2009; Chapter 12:Unit12.18. doi:10.1002/0471142956.cy1218s50

# Appendix: Microscope set up

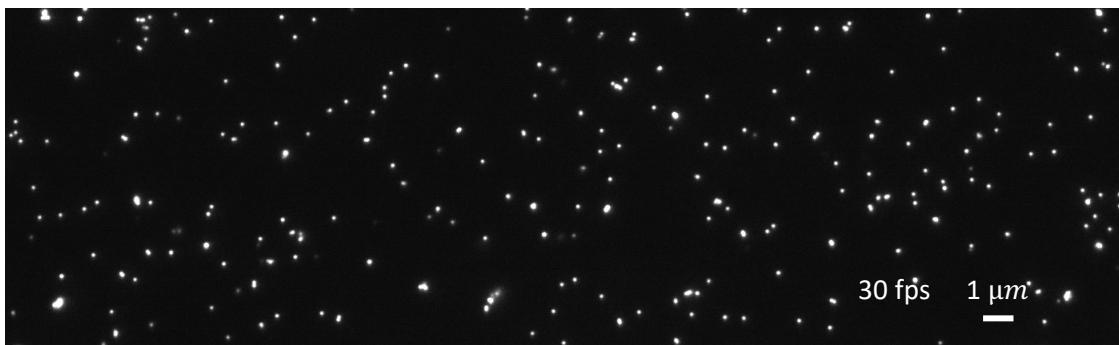


# Appendix

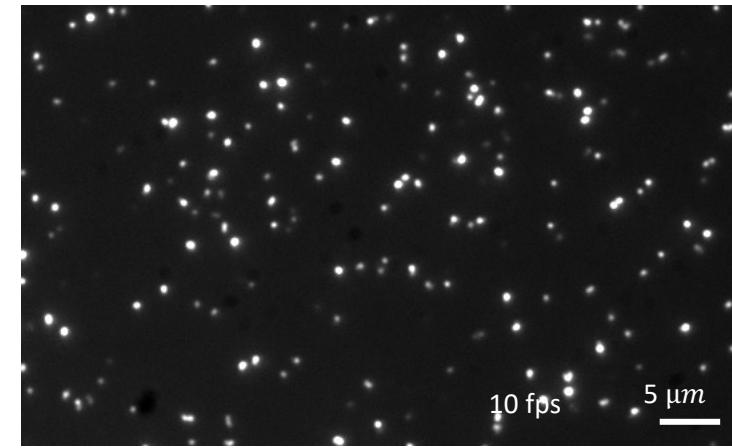
## MDE function for PSF calibration



# Appendix: TIRF vs Widefield



TIRF:  
'blinking' beads  
High contrast  
No out-of-focus beads visible



Widefield:  
Path of beads diffusing around  
Out-of-focus beads visible (farther from surface)

# Appendix

## ACF fitting function used in Quick Fit

This function implements a 1-component pure-diffusion model for data acquired with a camera in TIR illumination mode.[\[1\]](#) [\[2\]](#) The fit function is defined as:

$$g(\tau) = G_{\infty} + \frac{1}{N/A_{\text{eff}}} \cdot \frac{1}{a^2} \cdot \sum_{i=1}^3 \rho_i \cdot \left[ \text{erf} \left( \frac{a}{\sqrt{4D_i\tau + w_{xy}^2}} \right) + \frac{\sqrt{4D_i\tau + w_{xy}^2}}{\sqrt{\pi} \cdot a} \cdot \left[ \exp \left( -\frac{a^2}{4D_i\tau + w_{xy}^2} \right) - 1 \right] \right]^2$$

$$A_{\text{eff}} = \frac{a^2}{\left( \text{erf} \left( \frac{a}{w_{xy}} \right) + \frac{w_{xy}}{\sqrt{\pi} \cdot a} \cdot \left( e^{-\frac{(a/w_{xy})^2}{2}} - 1 \right) \right)^2}$$

The parameters are:

var.	Parameter
$G_{\infty}$	constant offset
$N$	particle number in the observation volume
$D_i$	$i$ -th diffusion coefficient
$\rho_i$	fraction of $i$ -th diffusing component
$a$	width of a (square) pixel in the image plane (i.e. pixel size of camera divided by the magnification)
$w_{xy}$	lateral radius of the point-spread function (PSF), $1/e^2$ -radius
$I$	average measured intensity in focus during measurement
$B$	average background intensity in focus during measurement

For the fractions we have in addition:

$$\rho_1 = 1 - \rho_2 - \rho_3$$

$$\Delta\rho_1 = \sqrt{(\Delta\rho_2^2 + \Delta\rho_3^2)}$$

$$\Delta A_{\text{eff}} = 0$$

$$\Delta C = \sqrt{\left( \frac{\Delta N}{A_{\text{eff}}} \right)^2 + \left( \frac{\Delta A_{\text{eff}} \cdot N}{A_{\text{eff}}^2} \right)^2}$$

For each component the mean squared displacement after a given time  $\tau_{MSD}$  is calculated, as:

$$\text{MSD}_i(\tau_{MSD}) = 6 \cdot D_i \cdot \tau_{MSD}$$

Also the time, required for a particle to leave the effective area of the pixel is calculated:

$$\tau_i(MSD) = \frac{A_{\text{eff}}}{4 \cdot D_i}$$