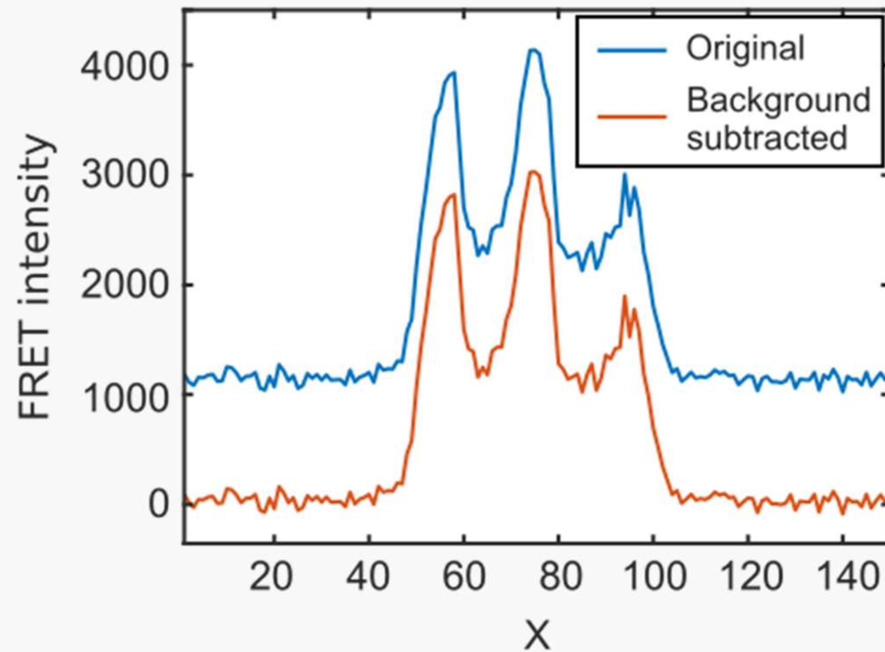


Lecture 9: Ratiometric imaging and background subtraction

MCDB/BCHM 4312/5312

Reminder: Mid-Term 1 is on Wednesday

- Bring
  - multiple pens/pencils
  - calculator (not your phone)

- You may prepare 1 side of 1 standard piece of paper (8.5” x 11”) with as many notes as you wish
  - 1 side, not 2

- For the mid-term, be sure to show all your work and pay attention to signs and syntax. Points will be lost.

- Exam starts at 11:30. You will have **80** minutes to take the exam.

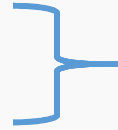
Just a Couple of Review Items

1. Nyquist
2. What is the difference between “Intensiometric” and “Ratiometric”?
3. FP Maturation
4. FRET

1. Nyquist

How often should you sample?

- At ½ the object size
- Or, at 2x the *Frequency*

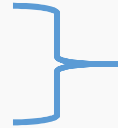


These say the same thing

1. Nyquist

How often should you sample?

- At ½ the object size
- Or, at 2x the *Frequency*



These say the same thing

So, if you have an object that is 100 nm in diameter, what is your sampling frequency?

A: 100 nm

B: 200 nm

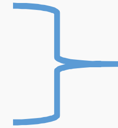
C: 50 nm

D: All of the above?

1. Nyquist

How often should you sample?

- At ½ the object size
- Or, at 2x the *Frequency*



These say the same thing

So, if you have an object that is 100 nm in diameter, what is your sampling frequency?

A: 100 nm

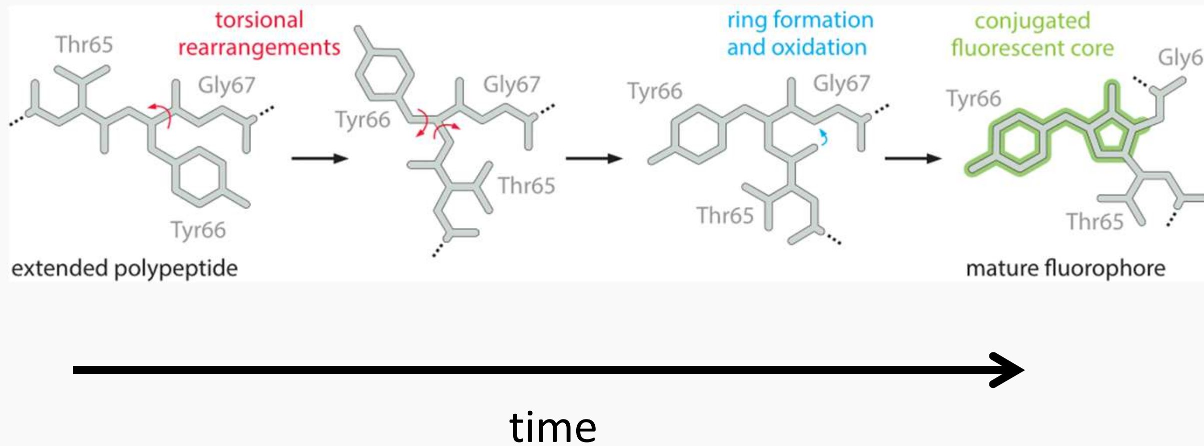
B: 200 nm

C: 50 nm

D: All of the above?

2. FP Maturation

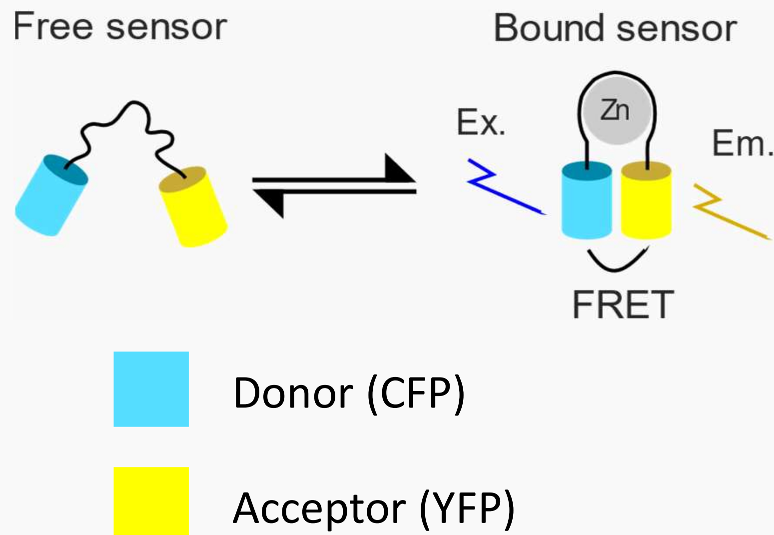
- Time it takes transition from its nascent state (non-fluorescent) into a fluorescent state

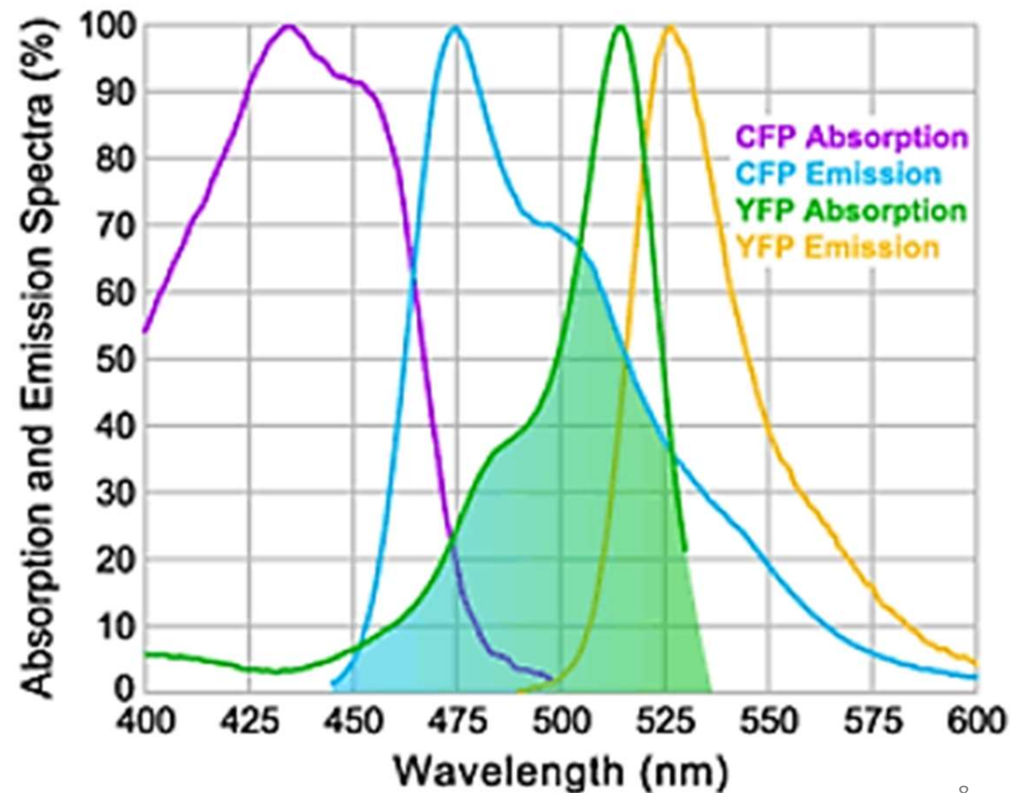


fluorophore	maturation time (min)	cell type
ECFP	50	*S. cerevisiae*
GFP wildtype	50	*in vitro*
sfGFP	6	*E. coli*
GFPmut3	7	*E. coli*
GFPmut3	7	*in vitro*
EGFP	60	*E. coli*
EGFP	14	*in vitro*
Emerald	12	*in vitro*
GFPem	5	*in vitro*
EYFP	40	*S. cerevisiae*
EYFP	20	*in vitro*
Venus	40	*in vitro*
mCherry	15	*E. coli*
mCherry	40	*S. cerevisiae*
mCherry	40–100	*E. coli*
mCherry	17 + 30	*S. cerevisiae*
mStrawberry	50	*E. coli*
tdTomato	60	*E. coli*
mPlum	100	*H. sapiens*, B-cell line

3. FRET

- Involves 2 fluorophores
  - A *Donor* fluorophore
  - An *Acceptor* fluorophore
- Needs a strong overlap between the *Donor* emission spectrum and the *Acceptor* excitation spectrum

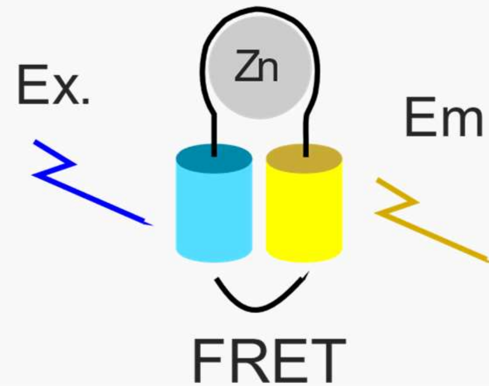


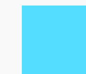


Ratiometric imaging

Free sensor

Bound sensor



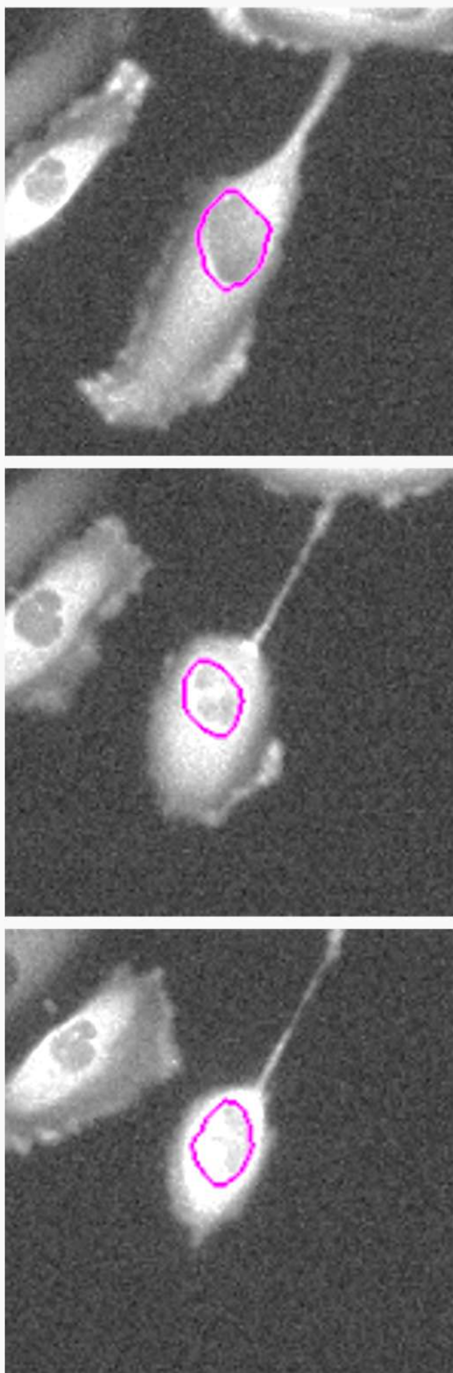
 Donor (CFP)

Acceptor (YFP)

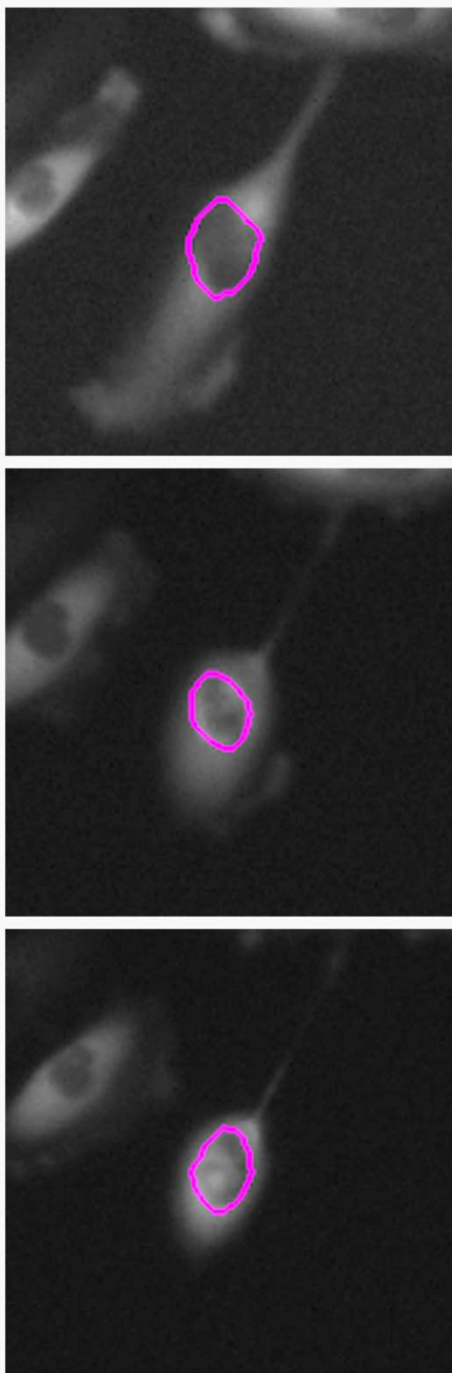
- How is the **FRET image** measured?
  - Excite with wavelength suitable for CFP
  - Collect the YFP emission

- How is the **CFP image** measured?
  - Excite with wavelength suitable for CFP (same as above)
  - Collect the CFP emission

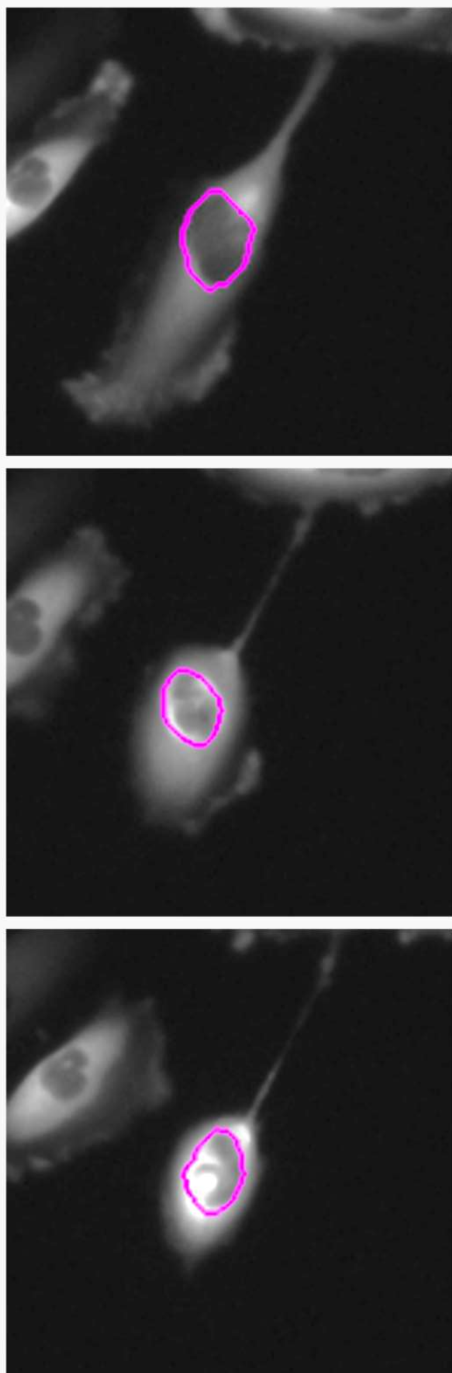
$$\text{Ratio} = \frac{\text{FRET}}{\text{ECFP}}$$



ECFP



FRET



Maria Lo, Palmer lab

4. FRET Principles

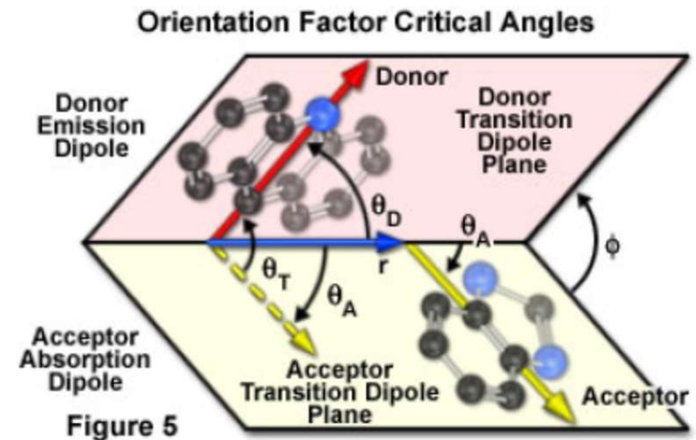
$$J = \int f_D(\lambda)\epsilon_A(\lambda)\lambda^4 d\lambda$$

f_D: Fluorescence of the Donor
ϵ_A: Extinction Coefficient of the Acceptor

Why is this important?

Q_o = QY of donor
κ = orientation factor
J = J(λ) = spectral overlap
n = index of refraction
N_A = avagardro's number

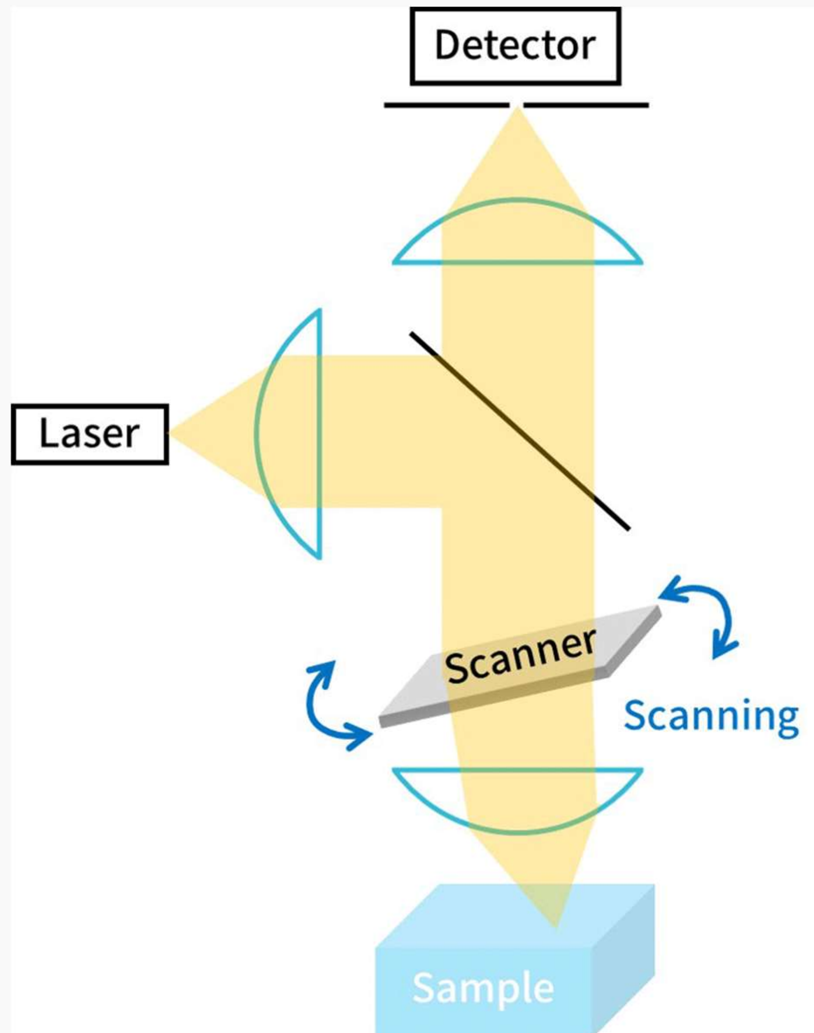
$$R_0^6 = \frac{9Q_0(\ln 10)\kappa^2 J}{128\pi^5 n^4 N_A}$$

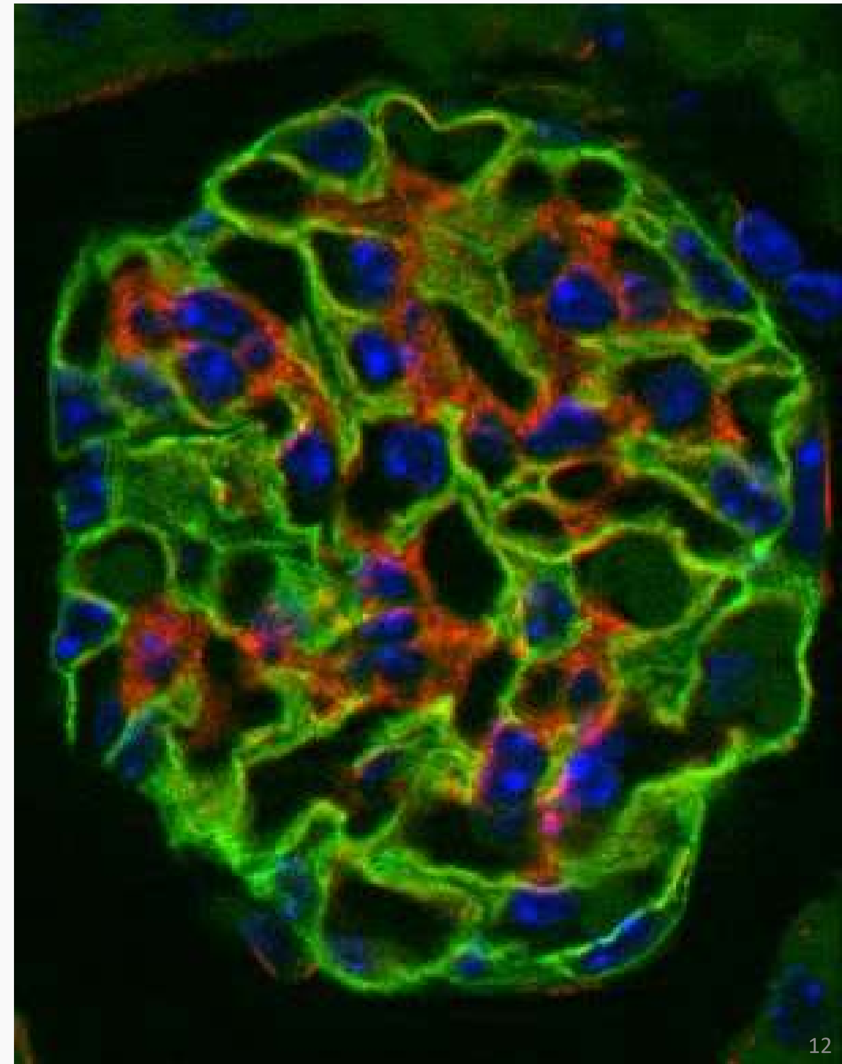


$\kappa^2 = (\cos\theta_T - 3\cos\theta_D\cos\theta_A)^2$
$= (\sin\theta_D \sin\theta_A\cos_\Phi - 2\cos\theta_D\cos\theta_A)^2$

Homework feedback

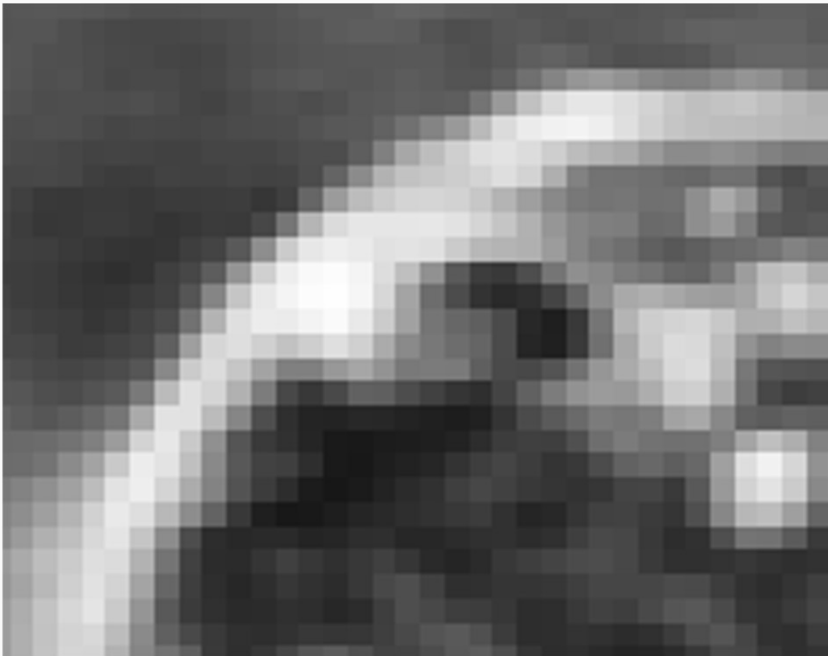
- Step sizes and pixel size for the laser scanning microscope





From Lecture 3:

Pixels are the measurement of intensity at a point in space rendered (typically) as squares



For a laser scanning microscope, the pixel size is equivalent to the "step size“ of the laser

Learning goals

- Correcting illumination in images
  - Sources of background illumination
  - Correcting for uneven illumination
  - Grayscale morphological operations to estimate background
  - Background subtraction
- Equations for computing the FRET ratio image

What are sensors?

- Fluorescent sensors are molecules that **change fluorescent intensity** when exposed to certain conditions, ions or molecules

- Sensors allow us to quantify some process based on intensity

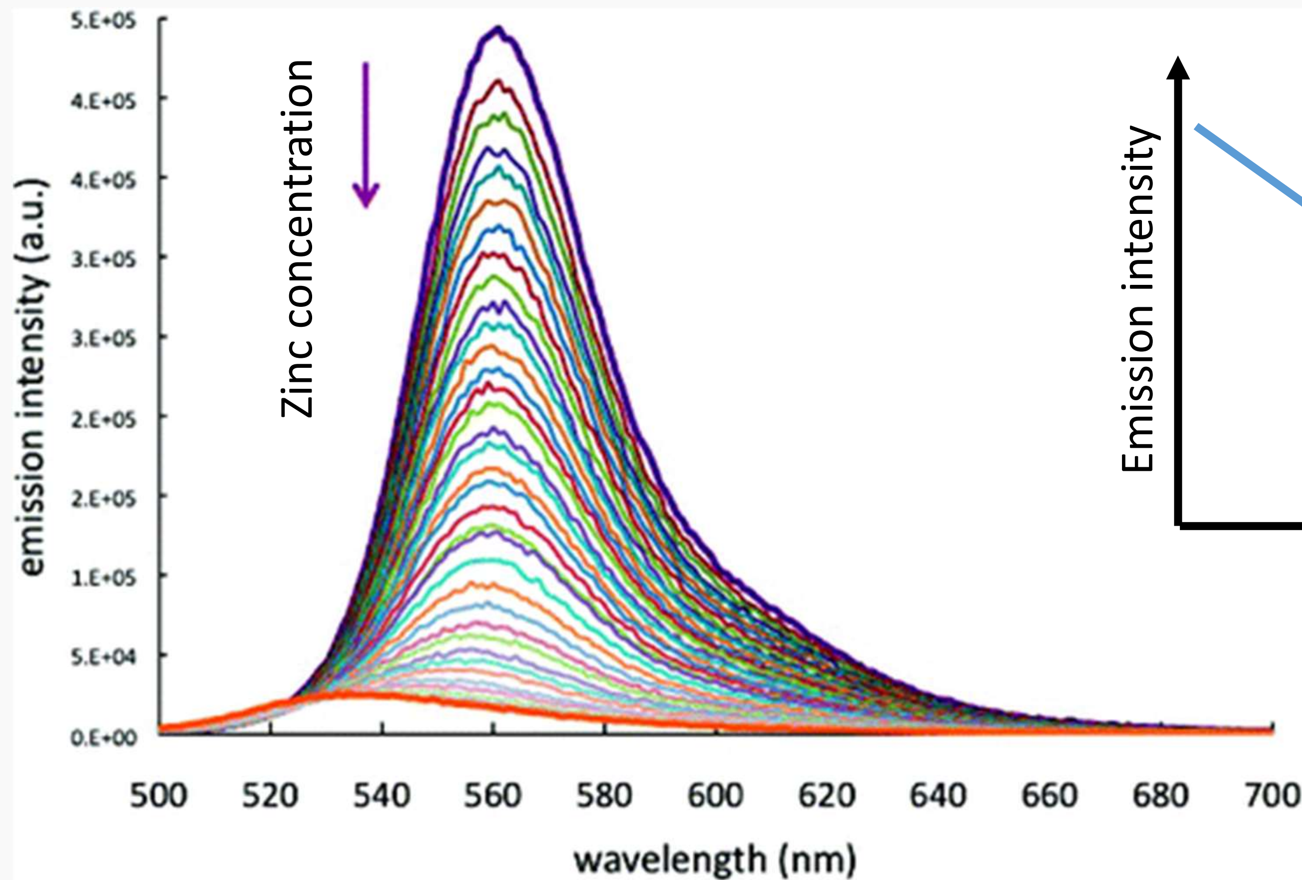
- Example is **metal-ion FRET sensors** from Evan's journal club:
  - Quantity of Ca2+ or Zn2+ ions in the cell – more ions, more fluorescence

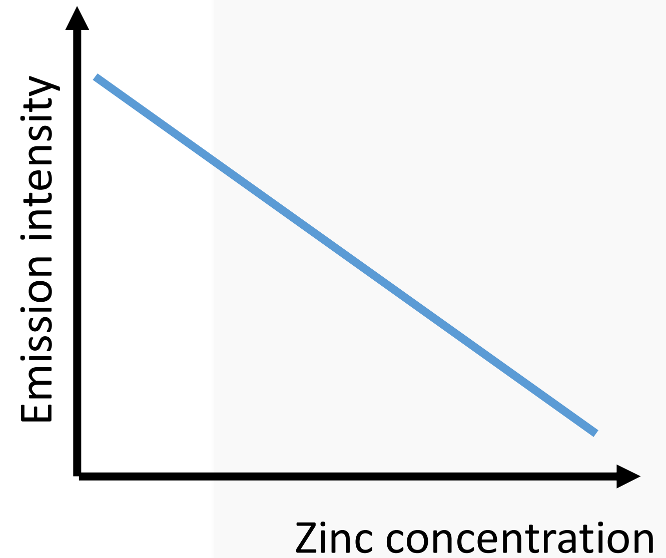
- But require proper calibration (i.e. measure quantity vs intensity) to be used for quantitative measurements
  - Rmax, Rmin measurements

Intensiometric measurements

- **Intensiometric = ΔIntensity**

Example: Zinc ion sensor – stops fluorescing when bound to zinc



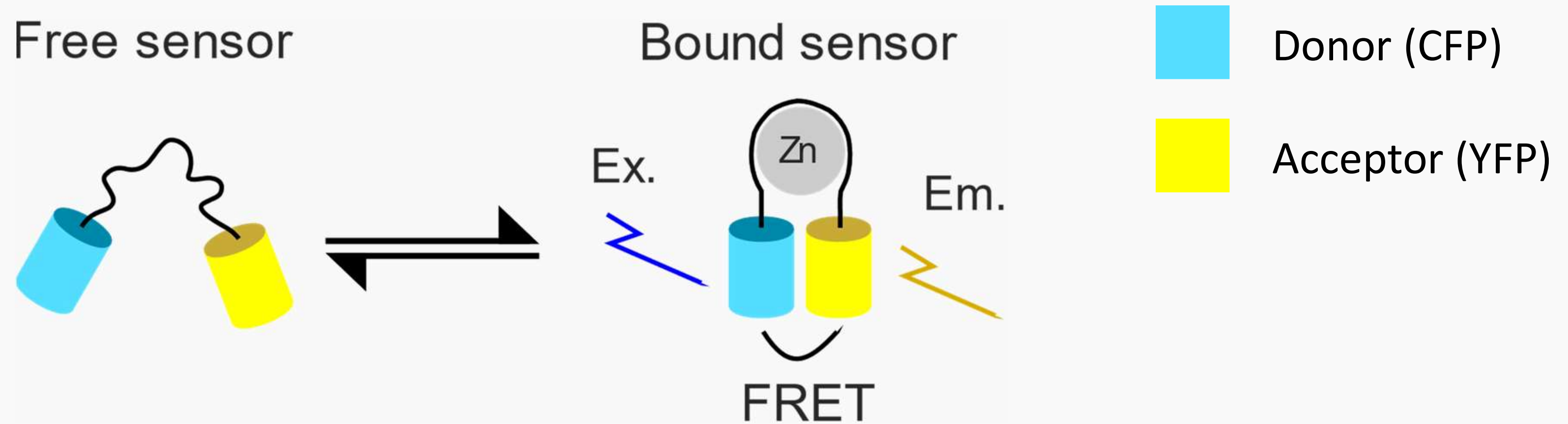


https://pubs.acs.org/doi/10.1021/ic101513a

What affects how bright a cell appears?

- Different amount of dye/fluorescent protein present in the cells
- Processes in the cell at time when image was taken
  - Cell dead or alive?
  - About to divide? In the process of division? Etc…
- Uneven excitation illumination
- Background signal
  - Autofluorescence
  - Dark noise
- We only want our sensor to be affected by conditions in the cell - **have to correct for all the other factors**

Ratiometric imaging



FRET ratio = FRET / CFP

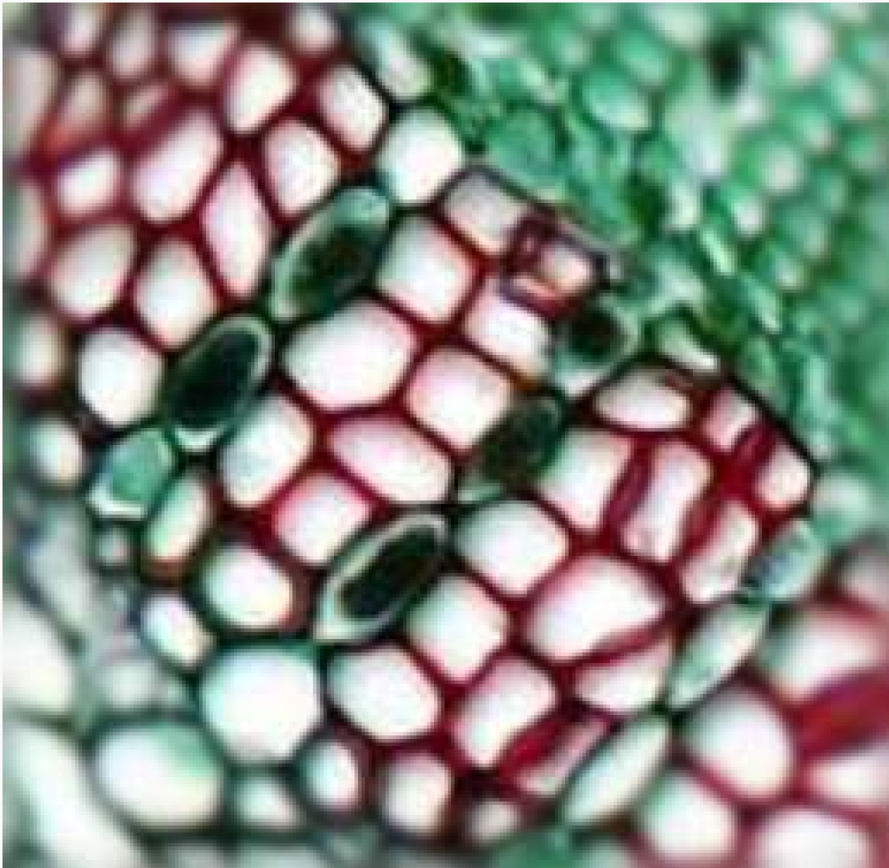
- What is the advantage of using the FRET ratio?
  - For FRET sensors, there is a fixed 1:1 ratio between number of donors (blue) and acceptors (yellow)
  - The ratio is not affected by the concentration of FRET molecules or photobleaching

Uneven excitation illumination

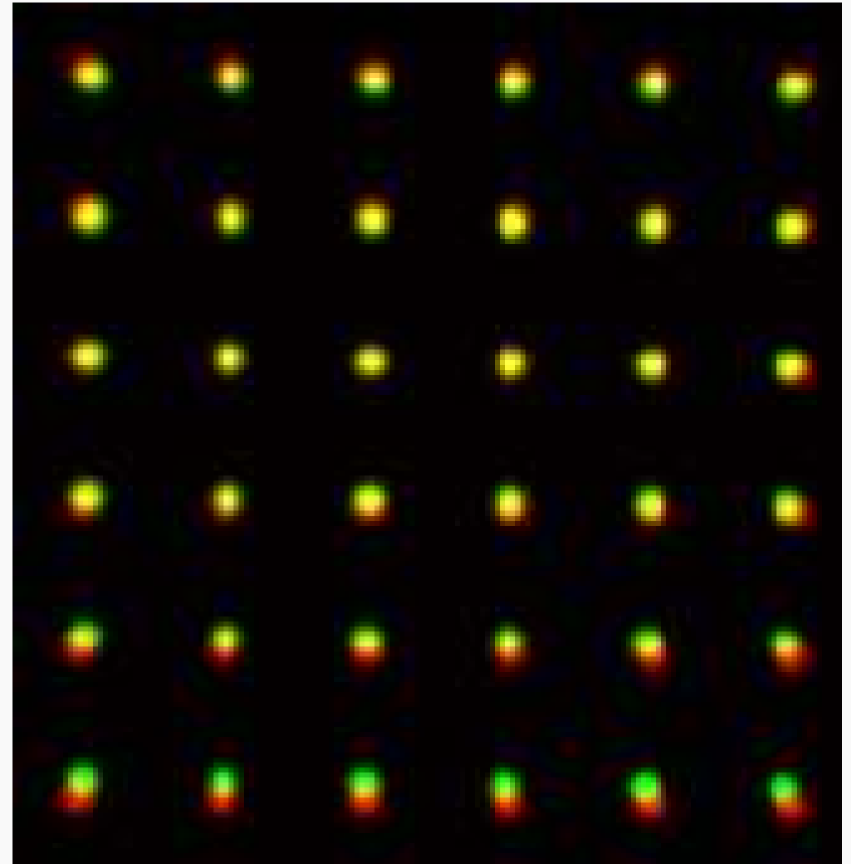
- Sources:
  - Misalignment of parts of the microscope
  - Damaged objectives or light guide (connects laser to microscope)
  - Flat field aberration – most common issue

Which of the following shows flat field aberration?

(A)



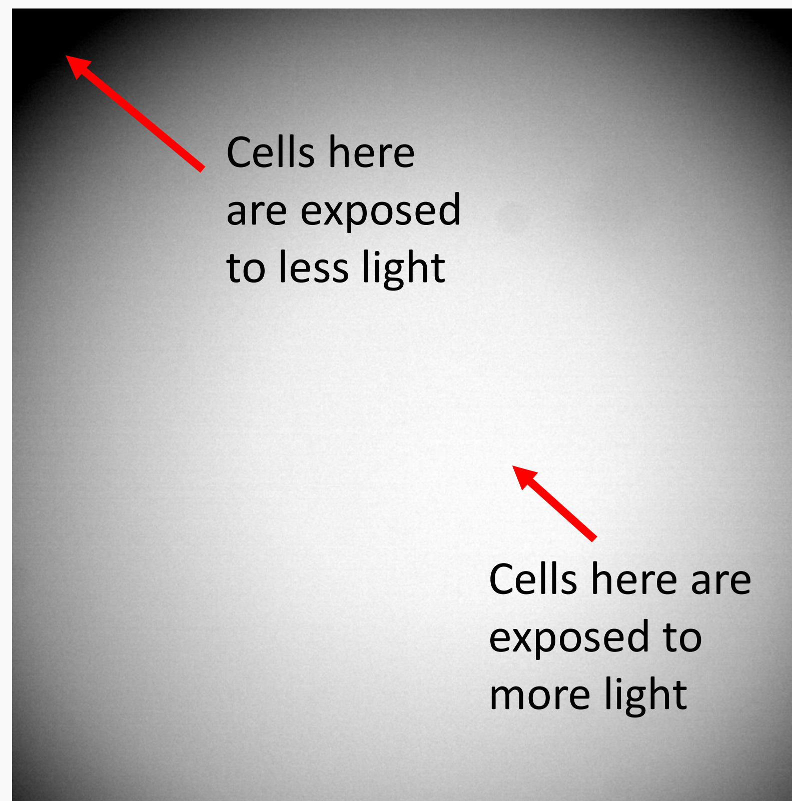
(B)



Measuring uneven illumination

Illumination pattern

Fluorescent slides

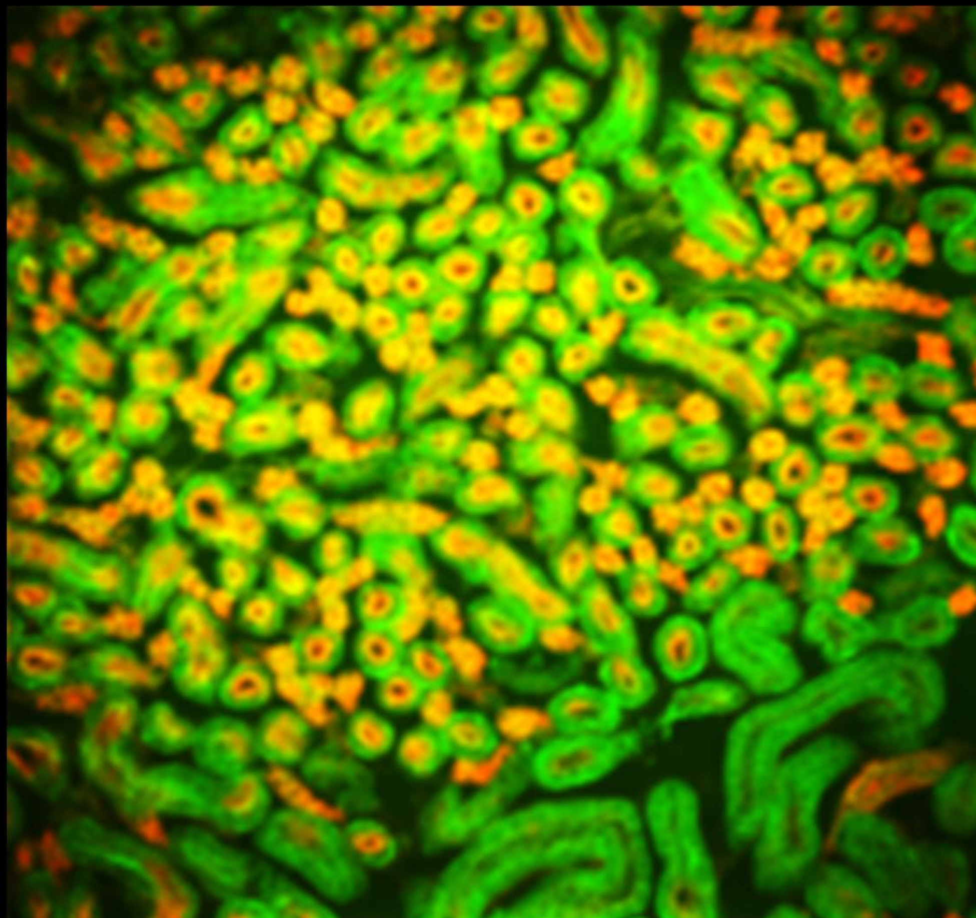


Widefield microscope, 10x objective

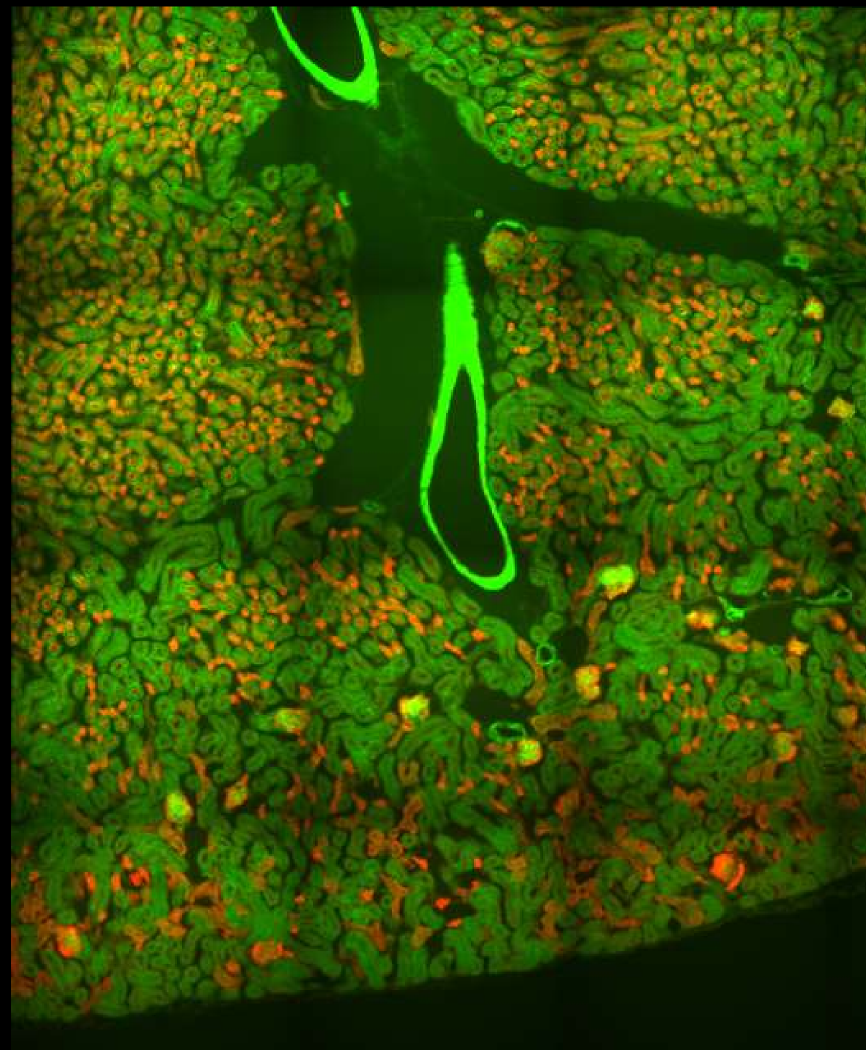
Uneven illumination

- Sources:
  - Misalignment of the microscope
  - Damaged objectives or light guide (connects laser to microscope)
  - Flat field aberration – most common issue

- Causes a position-dependent intensity pattern on the image



Tiled image consisting of
4 x 3 individual images



Uneven illumination

http://nic.ucsf.edu/blog/2014/01/shading-correction-of-fluorescence-images/

Correcting uneven illumination

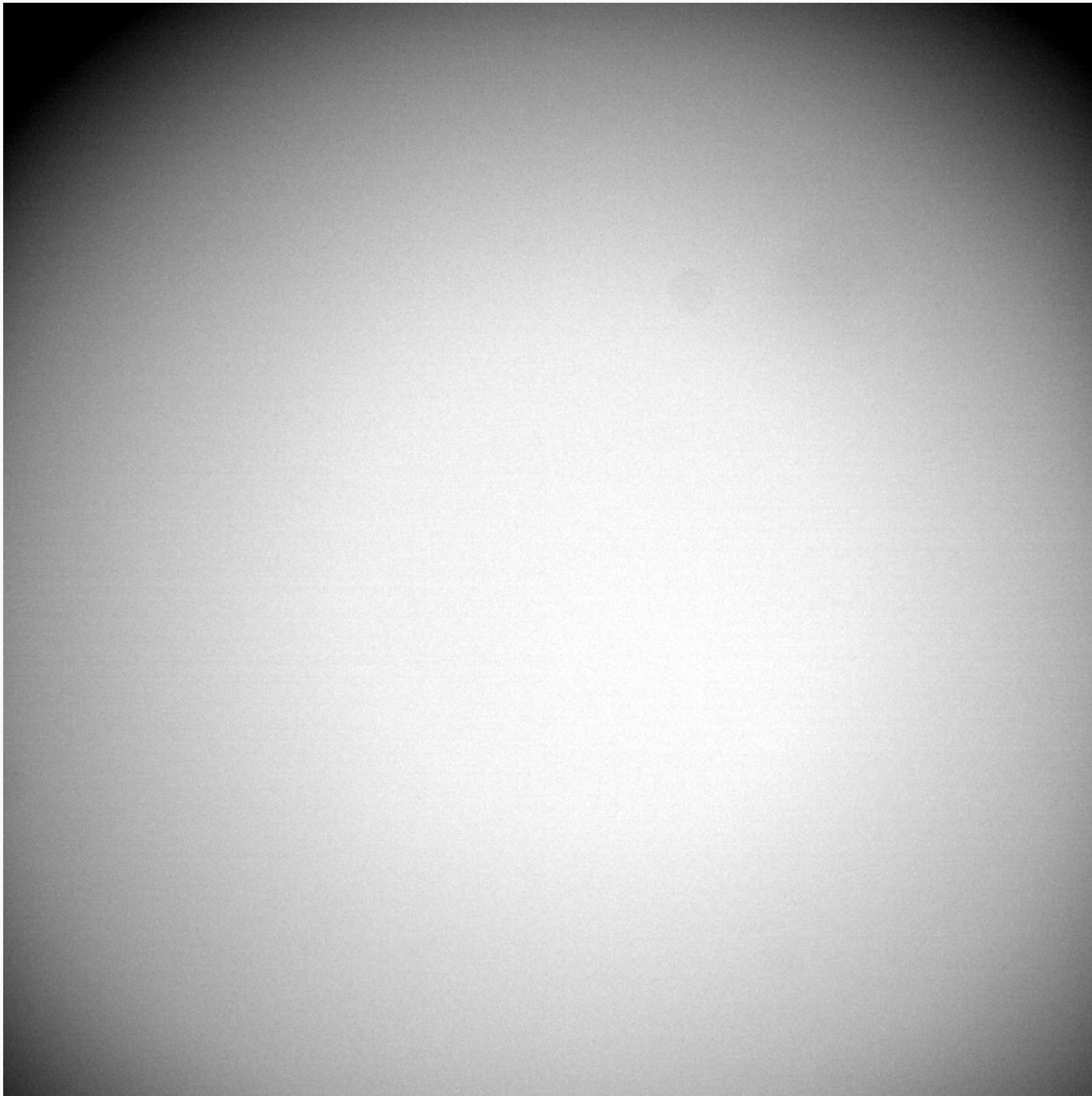
Step 1: Measure the illumination pattern using a fluorescent slide or thin uniform layer of concentrated dye

Step 2: Typically de-noise the image, using the Gaussian filter imgaussfilt

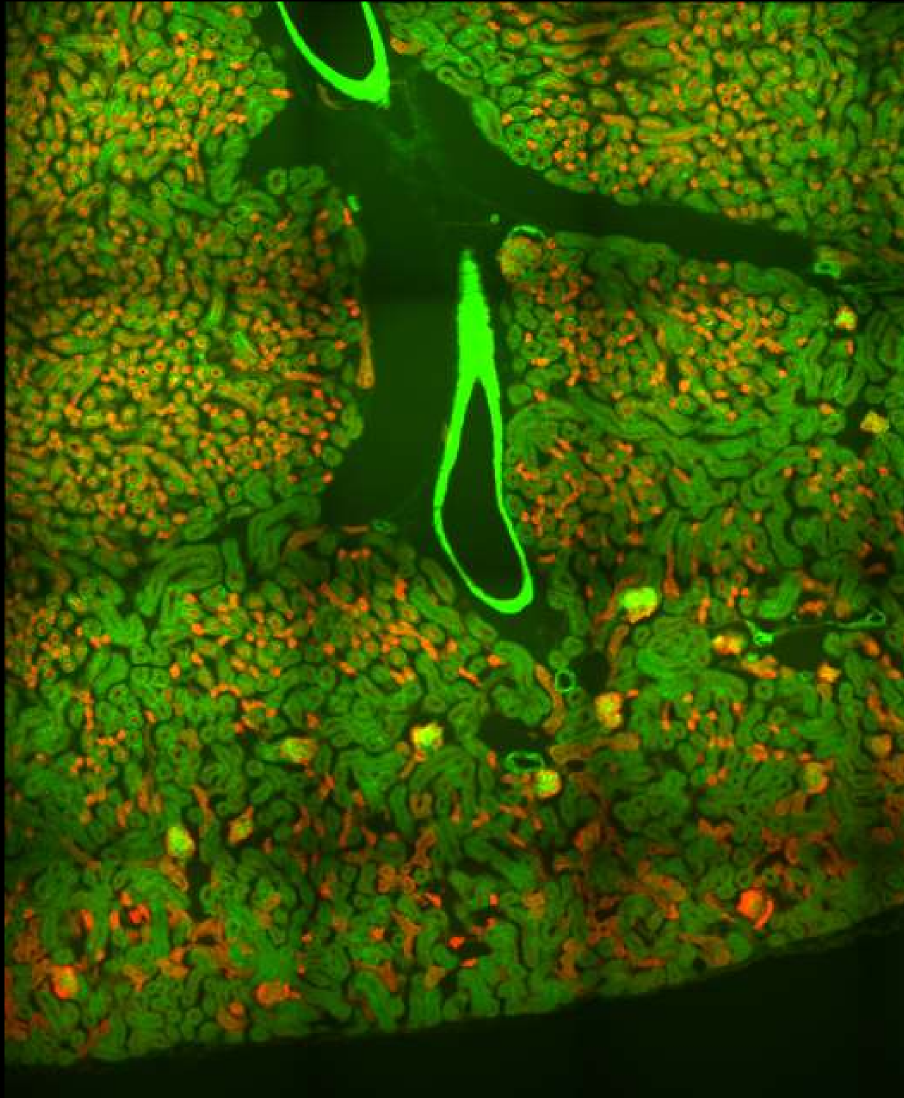
Step 3: Divide the fluorescent image by the illumination image

Why divide?

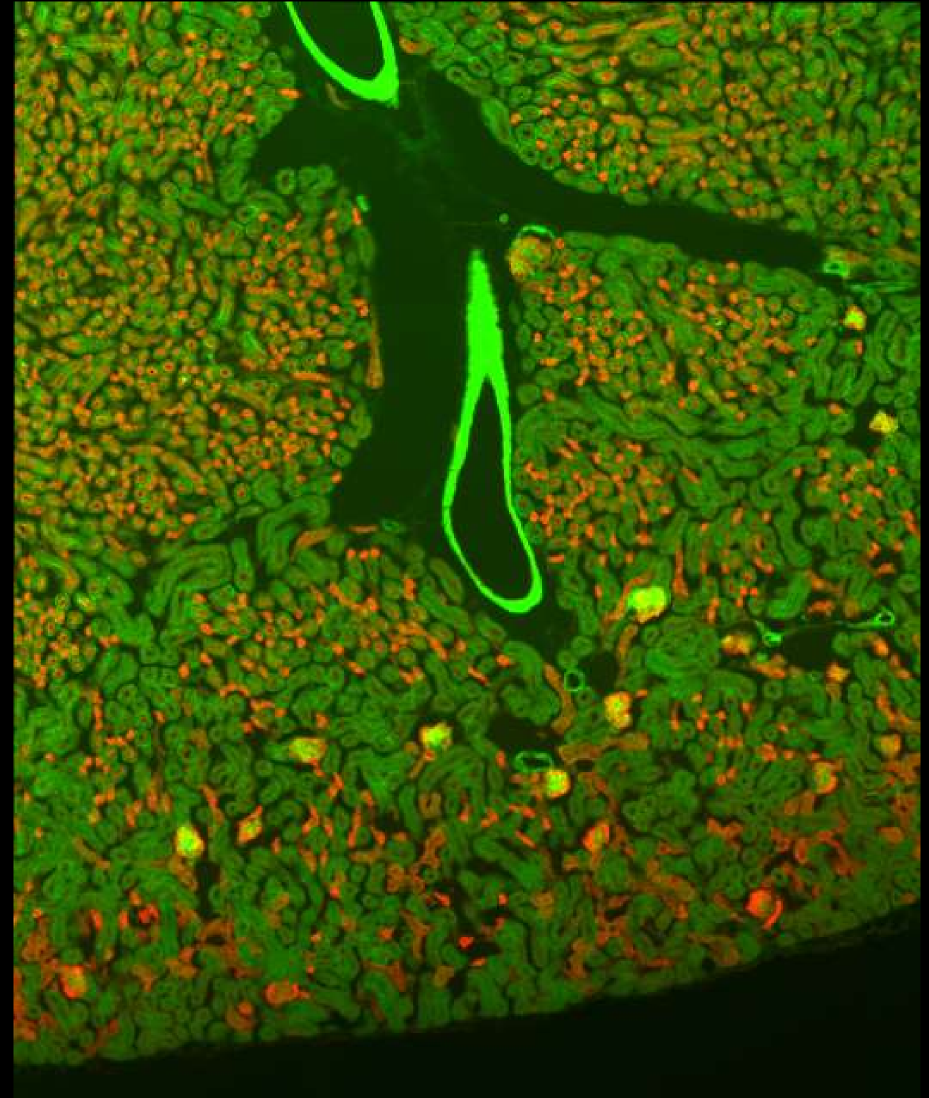
Fluorescence intensity is proportional to the excitation intensity



Tiled image consisting of 4 x 3 individual images



Uneven illumination



Corrected

http://nic.ucsf.edu/blog/2014/01/shading-correction-of-fluorescence-images/

MATLAB implementation

- Please try the practice question on Canvas

- I’ll give out the answer key on Monday

- **Don't forget to convert both images to doubles – you need the decimal places to get the correct ratio**

Background fluorescence

- Is caused by:
  - Fluorescence of the media surrounding the cell (e.g. growth media, mounting media)
  - Scattering from cells, agar pad, slide, bubbles etc.
  - Dark noise – inherent noise from the detector/camera
    - E.g. the image you take when you forget to remove the lens cap

Example dark noise image
thorlabs.com

Dark noise

- Every digital sensor/camera suffers from this
- Image this by capturing an image with no light on the sensor
  - Take a picture with the lens cap on
- Is affected by temperature of the sensor or camera chip
- Is uniform over the entire image

Background fluorescence

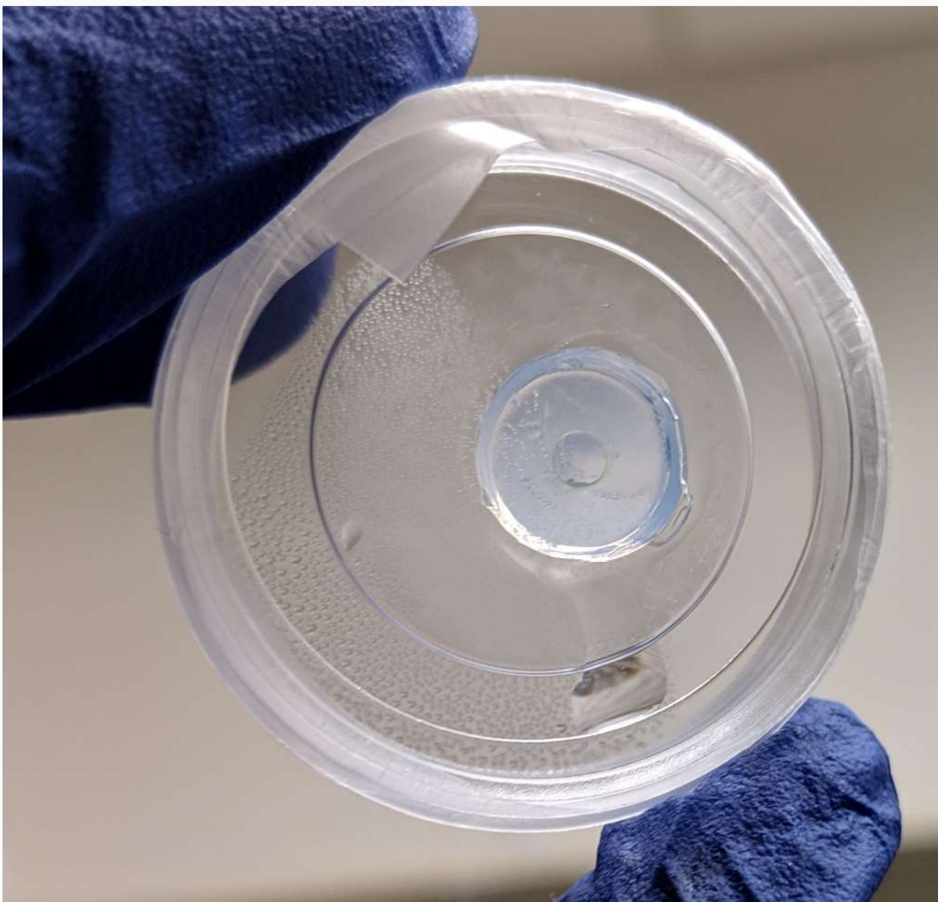
- Is caused by:
  - Autofluorescence of the media surrounding the cell (e.g. growth media, mounting media)
  - Scattering from cells, agar pad, slide, bubbles etc.
  - Dark noise – inherent noise from the detector/camera

- All these sources contribute light that **add up** with the signal from the image

Imaging background fluorescence

Best method:

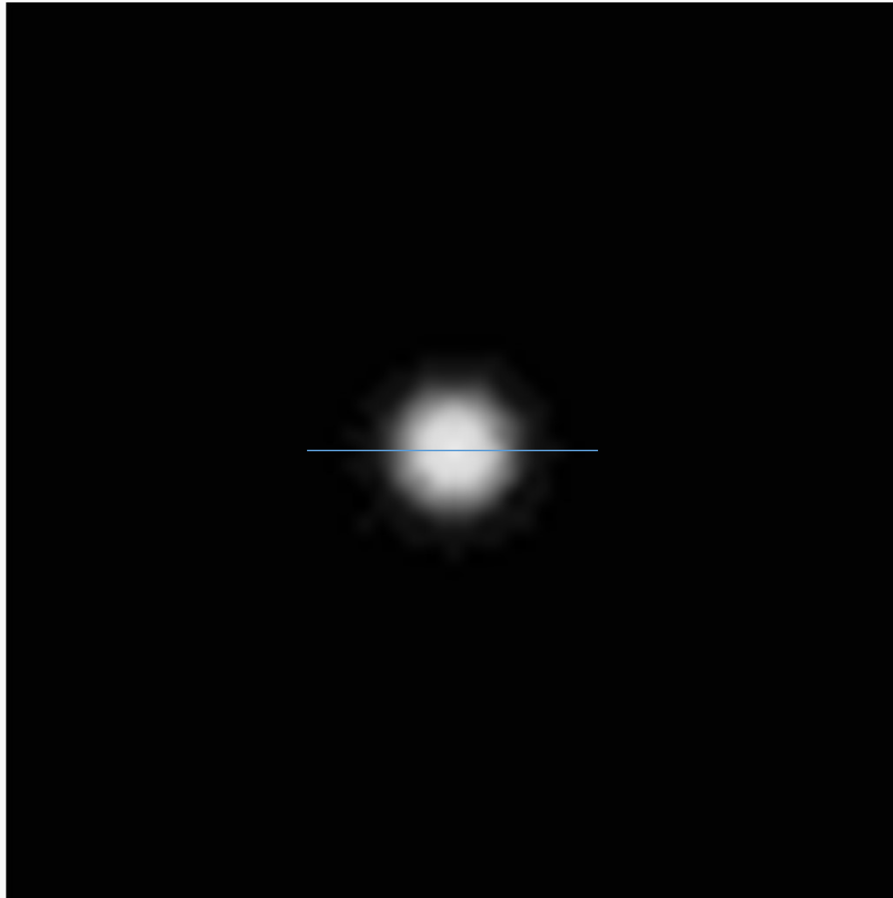
- Image a "blank" – sample with media/agar but no cells/fluorescent material

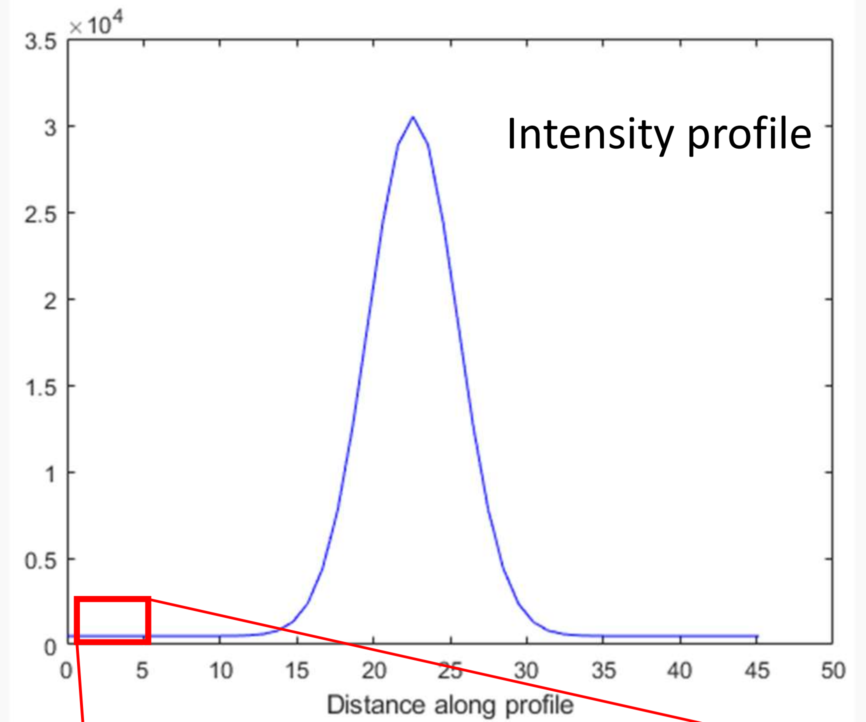


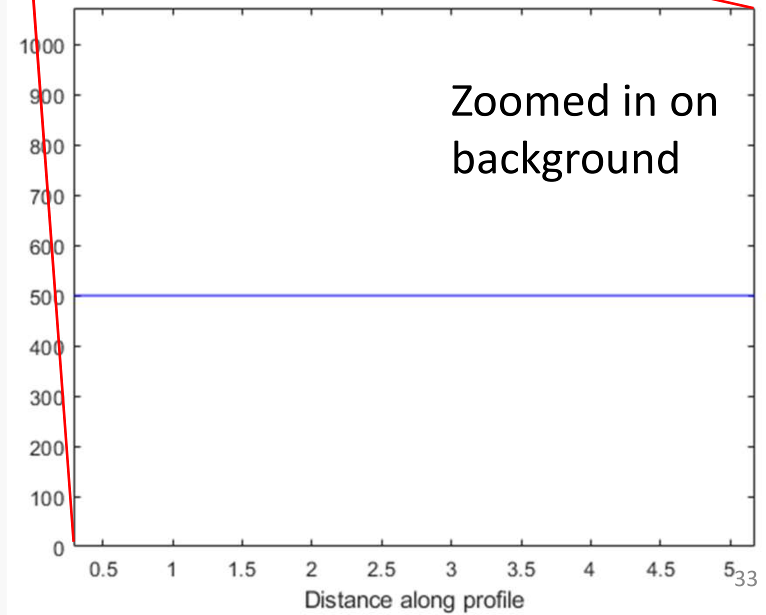
Imaging dish

Image a blank dish/with agar pad but no cells

From PS7

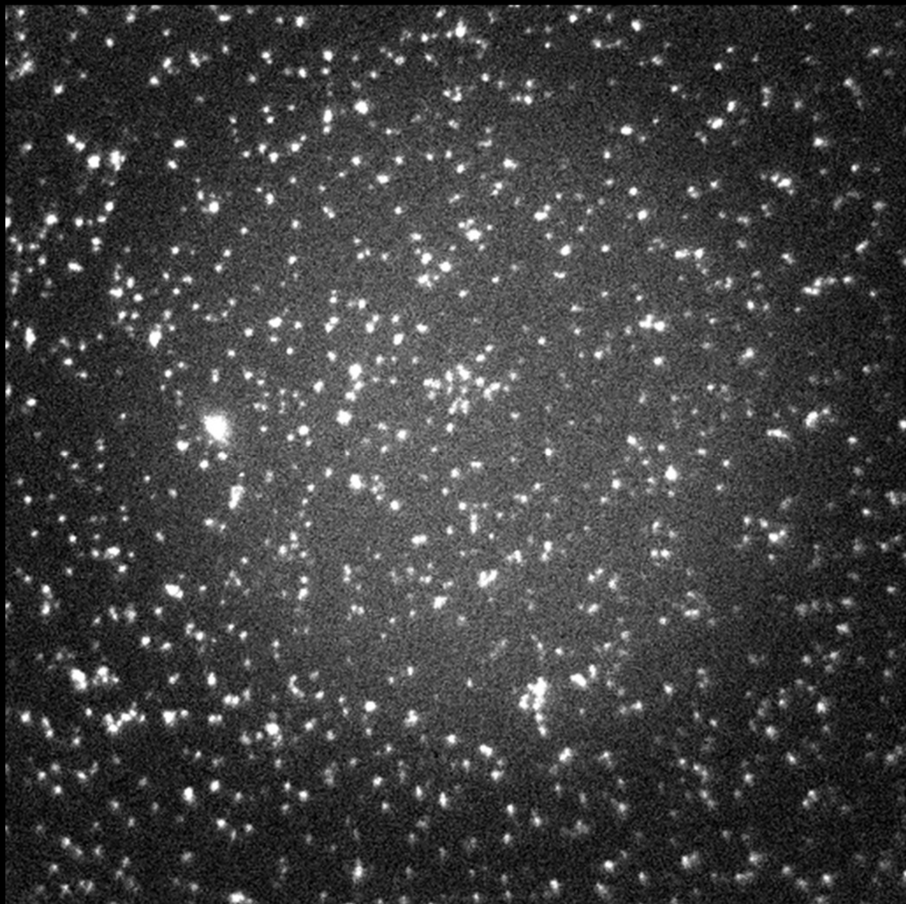




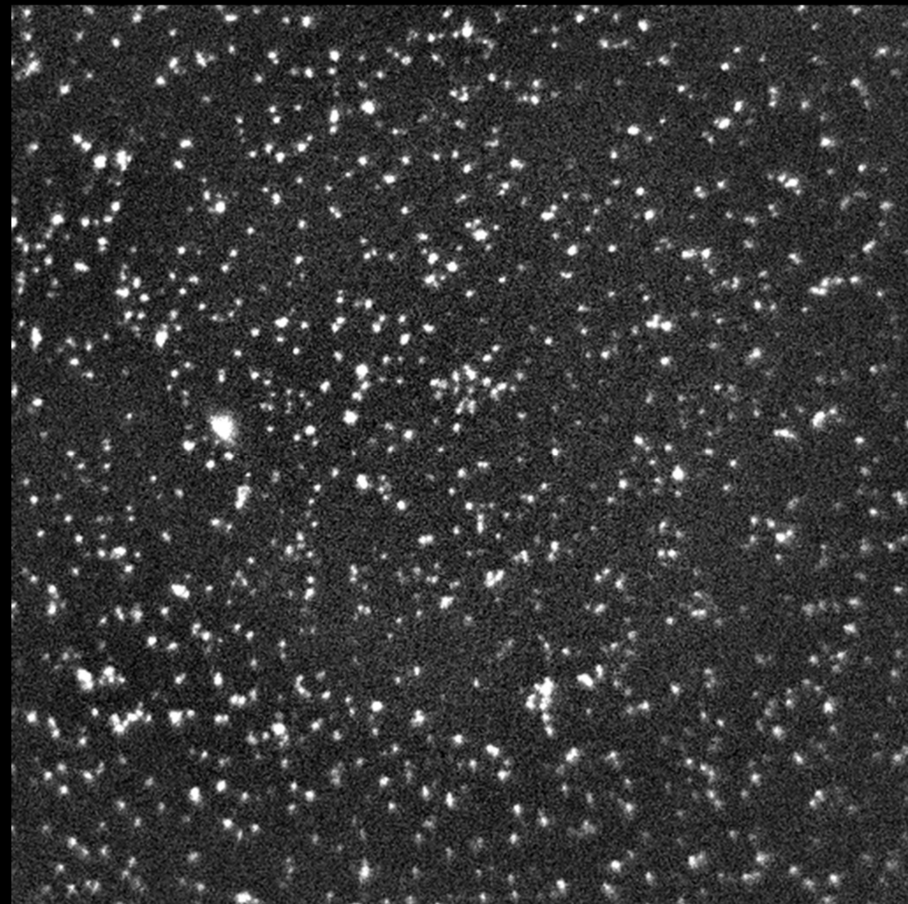


Correcting for background fluorescence

- Background fluorescence adds to the fluorescence from the cells
- Correct the background by SUBTRACTING background image from original



Background due to medium autofluorescence



After correction

http://help.imageanalyst.net/workflow_BackgroundSubtraction.html

Estimating the background

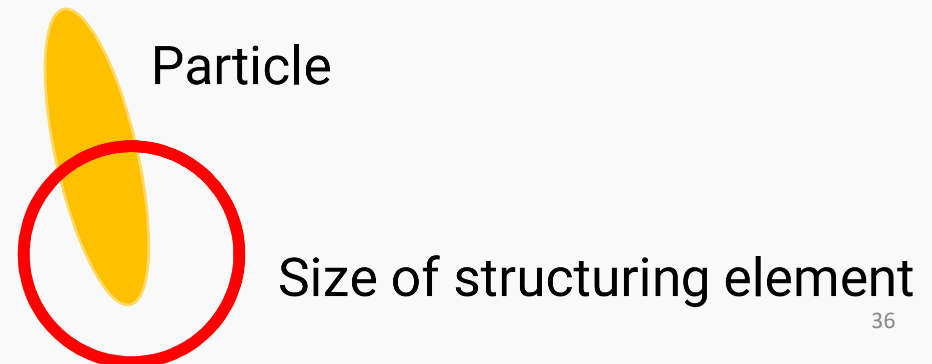
- To estimate the background, use grayscale morphological opening

- For grayscale morphological operations:

  - Erosion – minimum value in the neighborhood (true pixels) of the structuring element

  - Dilation – maximum value in the neighborhood of the structuring element

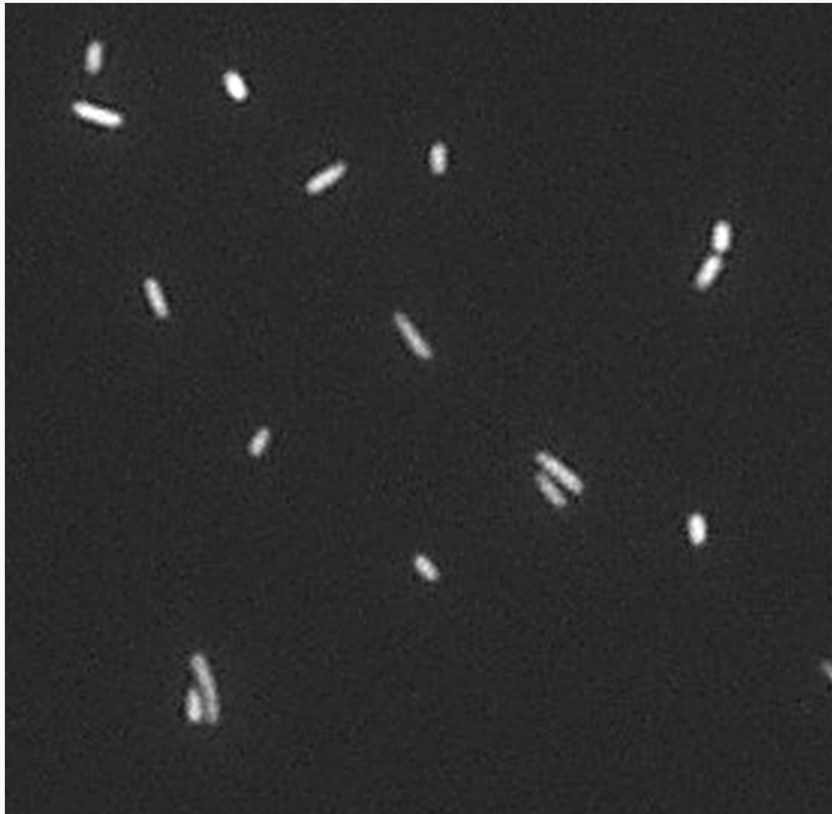
- The structuring element should be LARGER than your cells (or clusters of cells) because you want to measure the background value using the erosion step



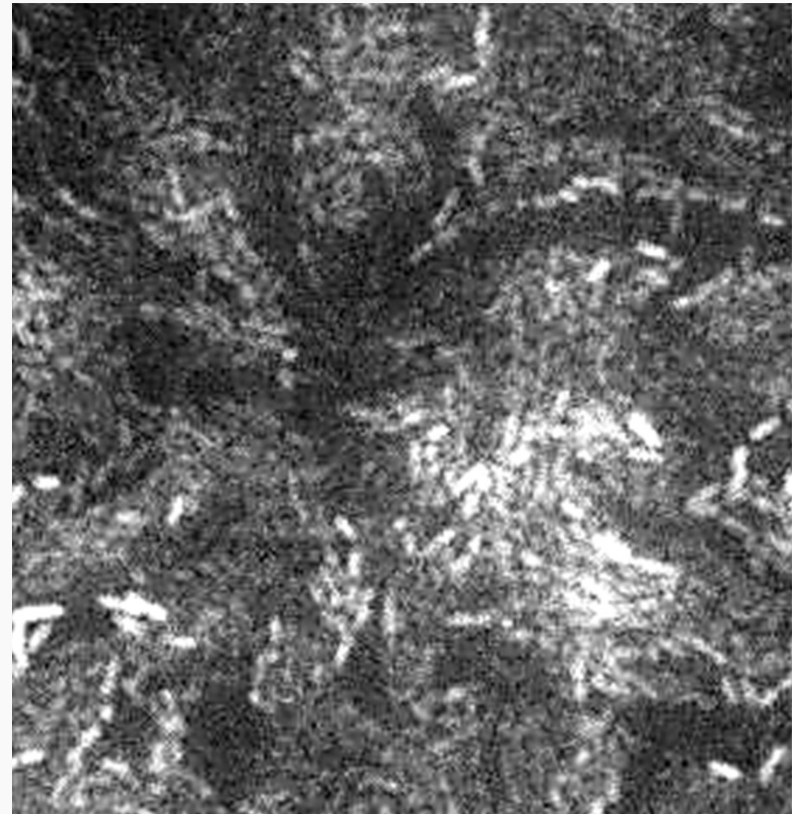
Estimating the background

- This approach only works when:
  - The image has background values – i.e. it is not fully crowded with cells
  - The cells are not in large colonies
  - Because there needs to be regions of just background
- Otherwise, the best thing to do is to take a background image

Suitable images for background estimation



This image works
Cells are well separated, there is plenty of background pixels to sample



This image does not work
There are too many cells, no background pixels

MATLAB example

1. Read in the image ‘rice.png’. Note that the image has an uneven background

2. Estimate the background by using imopen. Choose the structuring element size carefully – must be larger than the rice grains. A disk shape will work fine

3. Smooth the image using the Gaussian filter

4. Subtract the background

MATLAB code

```
I = imread('rice.png');

%Display the image
imshow(I, [])
%Use the distline tool to estimate the size of the structuring %element
imdistline

%Grayscale opening to estimate the background
bg = imopen(I, strel('disk', 15));

%Smooth the background with a Gaussian filter
bg = imgaussfilt(bg, 3);
imshow(bg, []) %Plot to check

%Subtract the background
Icorr = I - bg;
imshow(Icorr, [])
```

A more complicated way of estimating the background

- Segment your cells, then look at the datapoints in the background image

- Fit the background to a 2D Gaussian surface (we'll hopefully look at this in a couple of weeks)

Take away points

- For quantitative fluorescence measurements, correct for uneven and background illumination

- Background fluorescence
  - Measure or estimate the background intensity pattern
  - **SUBTRACT** it from the image

- Uneven illumination
  - Measure the illumination pattern using a slide or uniform layer of dye
  - **DIVIDE** it from the image (after subtraction)

- **Don't forget: Convert the images to double before performing the operations above**

Computing the FRET ratio

$$I_{FRET\ (corr)} = \frac{I_{FRET} - I_{FRET\ (bg)}}{I_{illum}}$$

$$I_{CFP\ (corr)} = \frac{I_{CFP} - I_{CFP\ (bg)}}{I_{illum}}$$

$$FRET\ ratio = \frac{I_{FRET\ (corr)}}{I_{CFP\ (corr)}}$$

Comparing fluorescent images

- When comparing different images, the assumption is that the excitation illumination has the same intensity each time

- BUT light sources behave differently due to humidity, temperature etc.
  - Measure everything on the same day (if possible)

- If not, best approach is to have a fiducial marker – some sample that you know will fluoresce the same way each time, e.g.
  - Well filled with thin layer of dye with a known concentration
  - Fluorescent beads

- DIVIDE by fiducial marker intensity in each image to normalize

Homework feedback

- Use : when you know the spacing you want

```
xdata = 0:0.05:(21 * 0.05)
```

or

```
xdata = (1:numel(ydata)) * 0.05
```

- Use linspace when you know how many points, but don't know/don't care about the spacing between the points