Lecture 25: Intensity corrections for quantitative imaging

University of Colorado Boulder

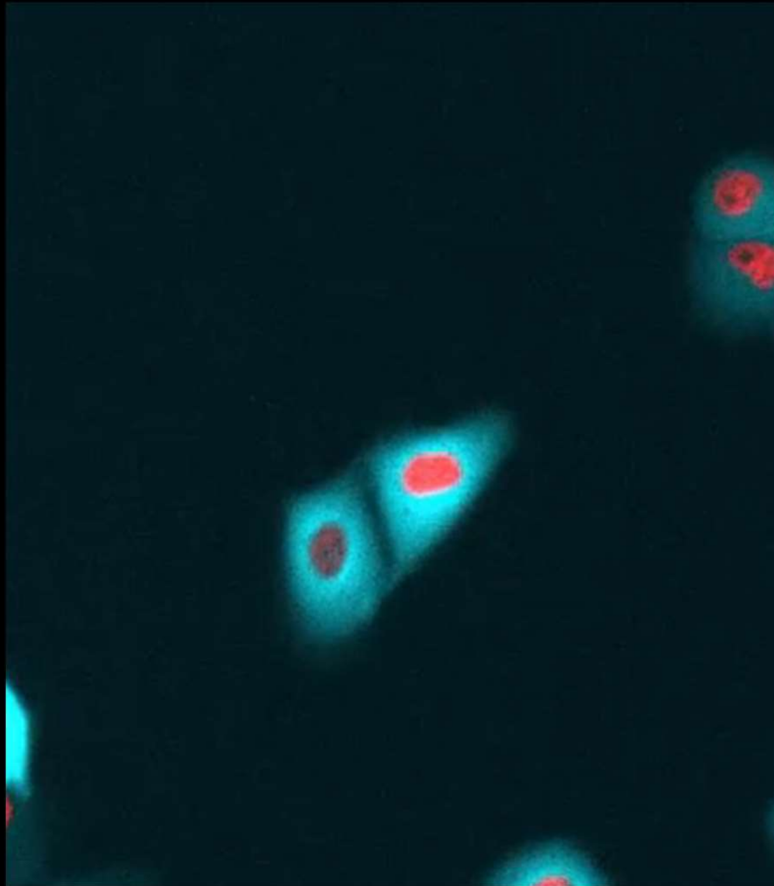
MCDB/BCHM 4312/5312
Fall 2020

In fluorescence microscopy,

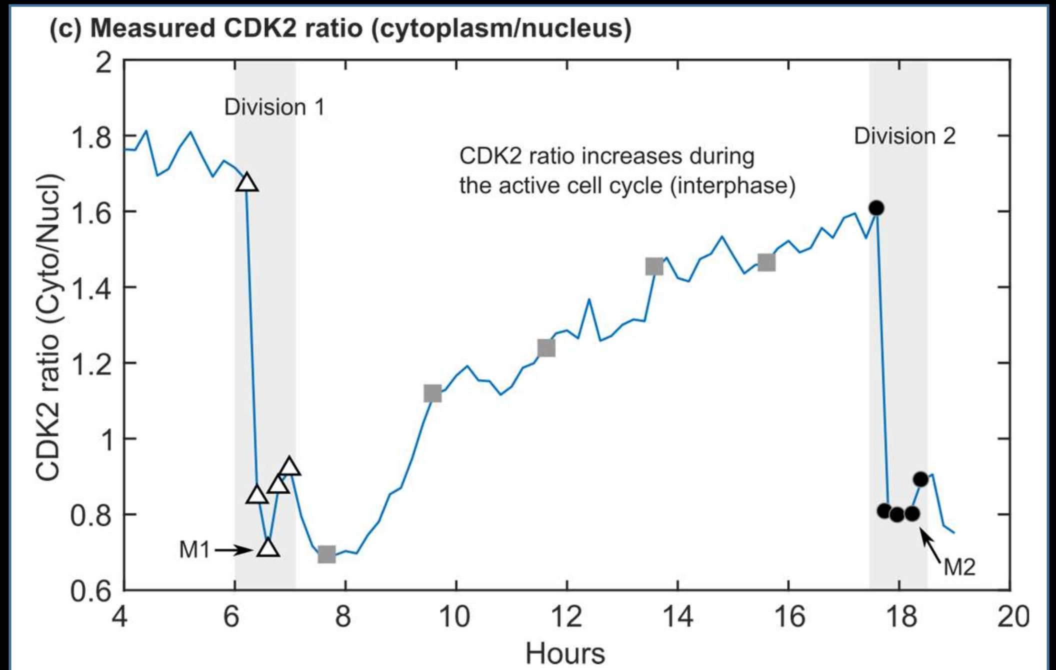
intensity is used to quantify cell activity

Fluorescence shows DNA repair after damage

J. Mahadevan, J. Rudolph, J. W. Tay, Karolin Luger, Biophys. J. **116**, 2224 (2019)

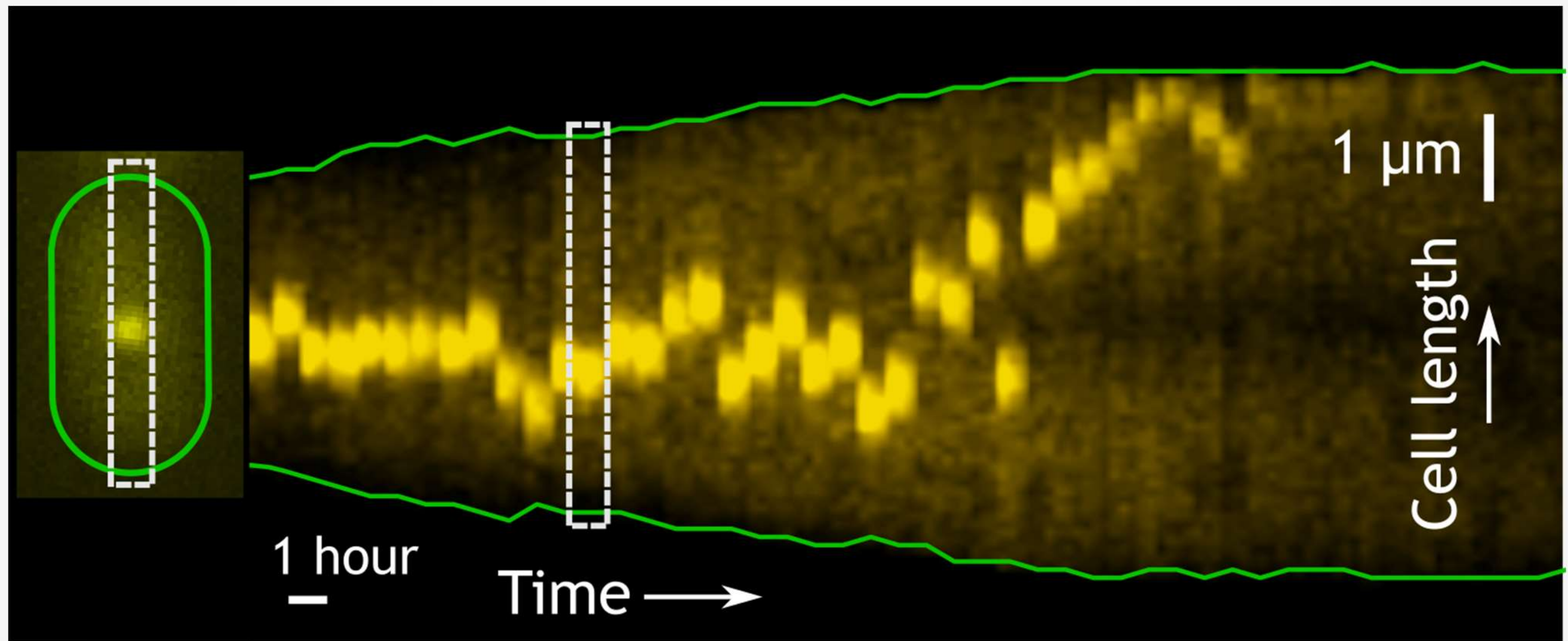


Fluorescence indicates when cells are going to divide



M. Lo, J. W. Tay, A. E. Palmer, eLife 9, e51107 (2020)

Track position of organelles with time and cell activity



Nicholas C. Hill, Jian Wei Tay, … Jeffrey C. Cameron *Science Advances* **6**, eaba1269 (2020)

Using regionprops to measure intensity

```
data = regionprops(mask, image, 'MeanIntensity')
```

First input argument is always the mask

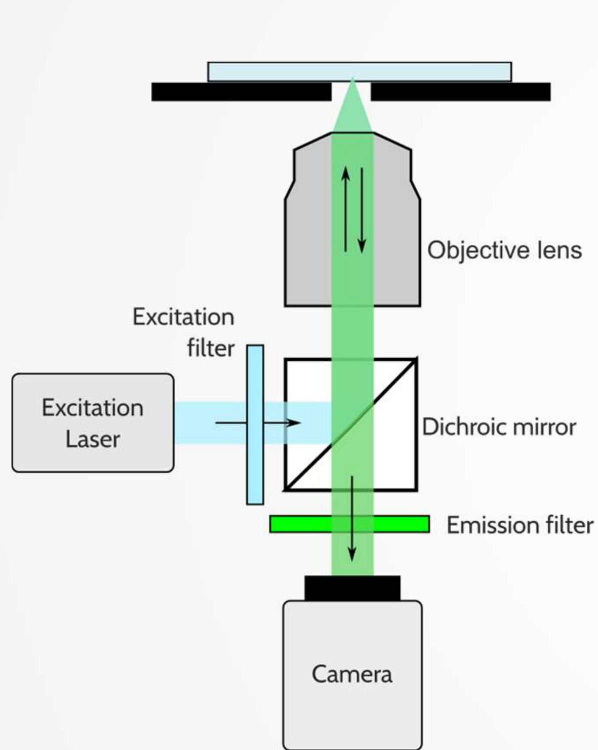
Second input argument is the image

Two common problems with real life imaging

- Uneven illumination
- Background fluorescence

Uneven illumination

The excitation laser has a spatially-dependent intensity pattern



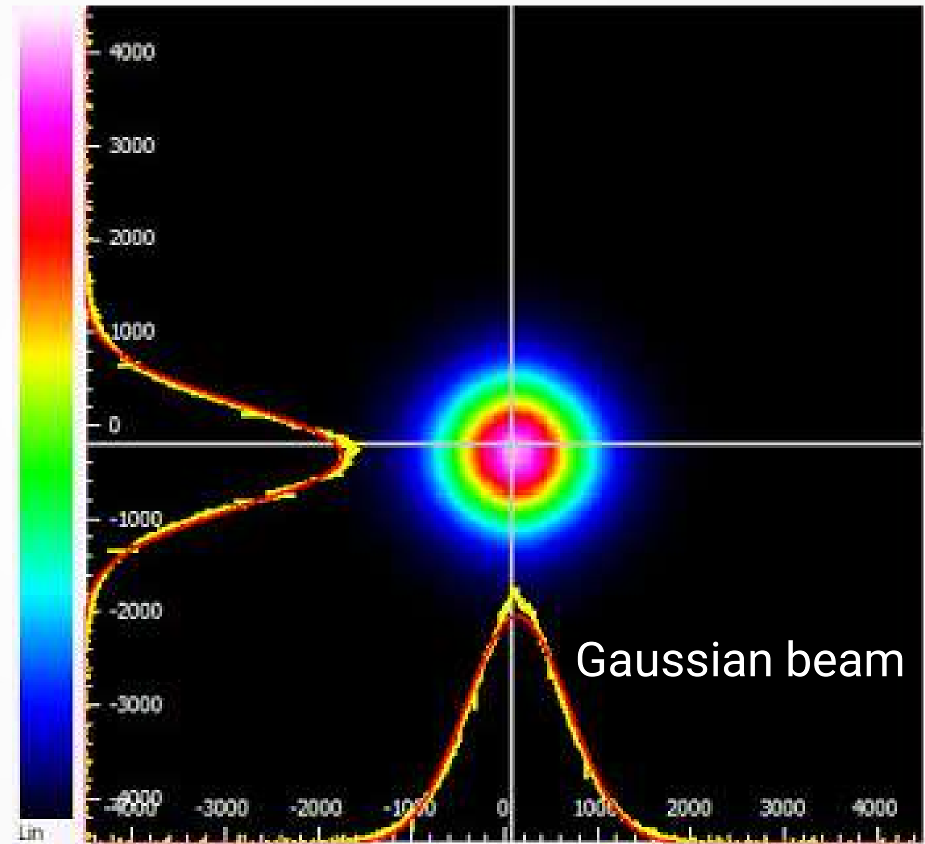
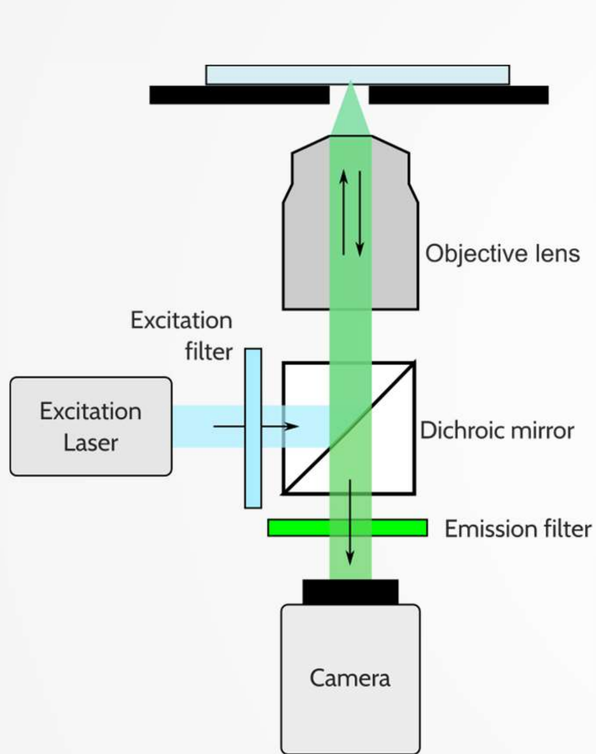


Image stolen from the internet

The excitation laser has a spatially-dependent intensity pattern



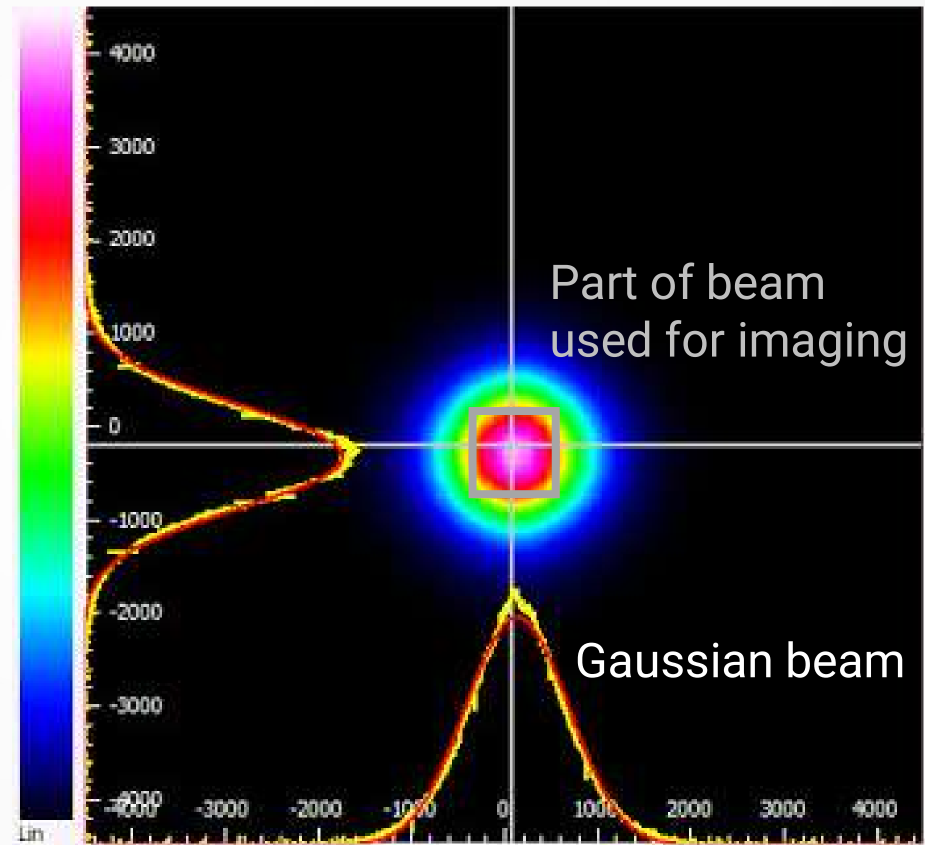
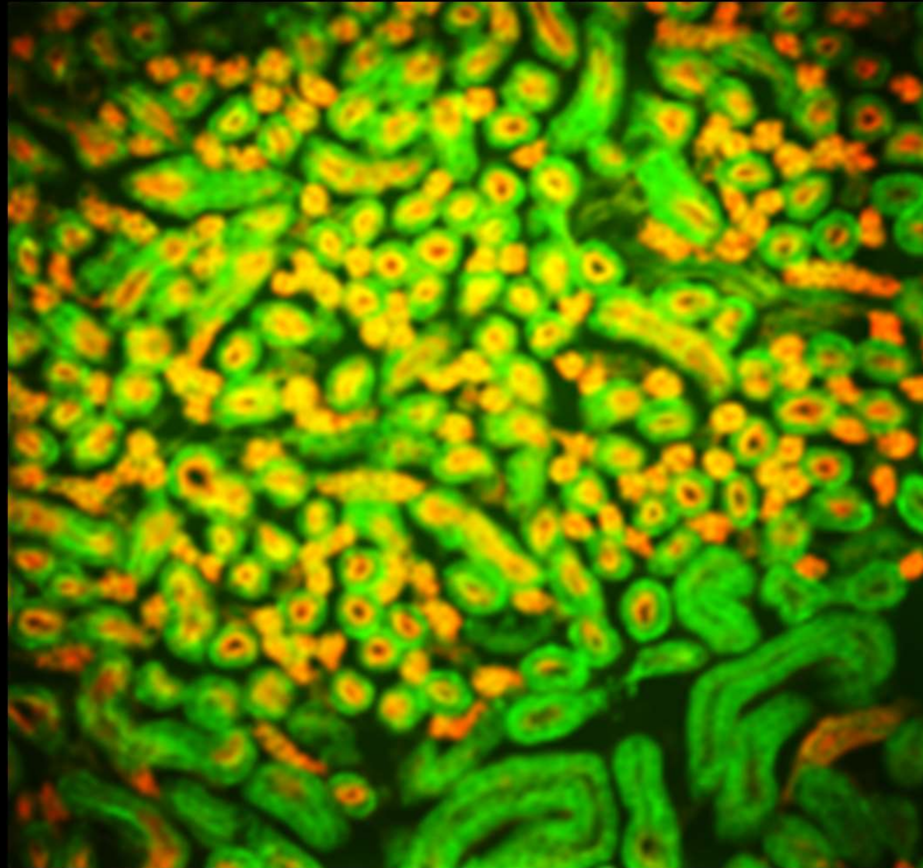
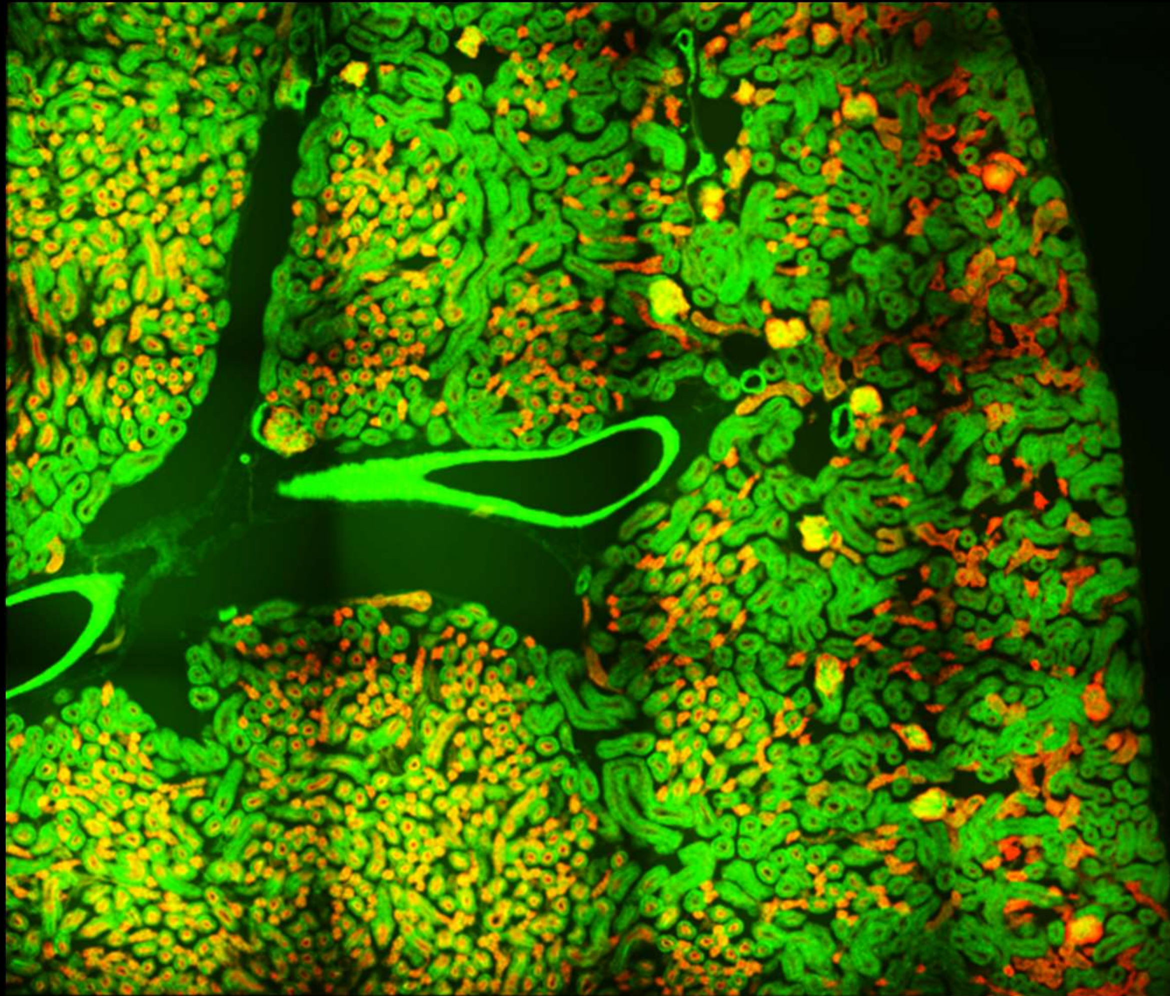


Image stolen from the internet



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

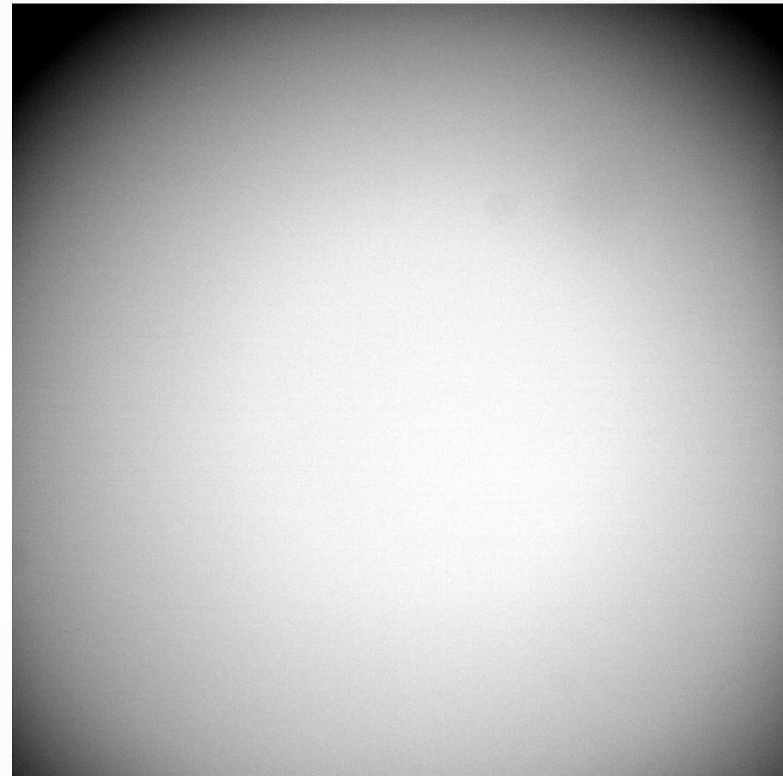


Tiled image consisting of
4 x 3 individual images

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

Measuring uneven illumination

Fluorescent slides



Calibration image captured on a widefield microscope, 10x objective

What is the effect of uneven illumination?

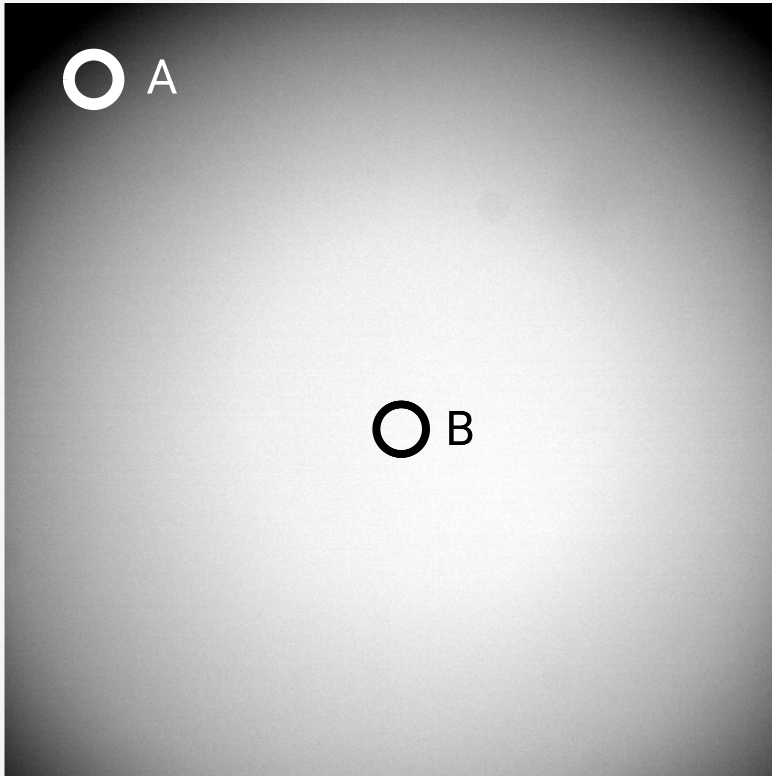
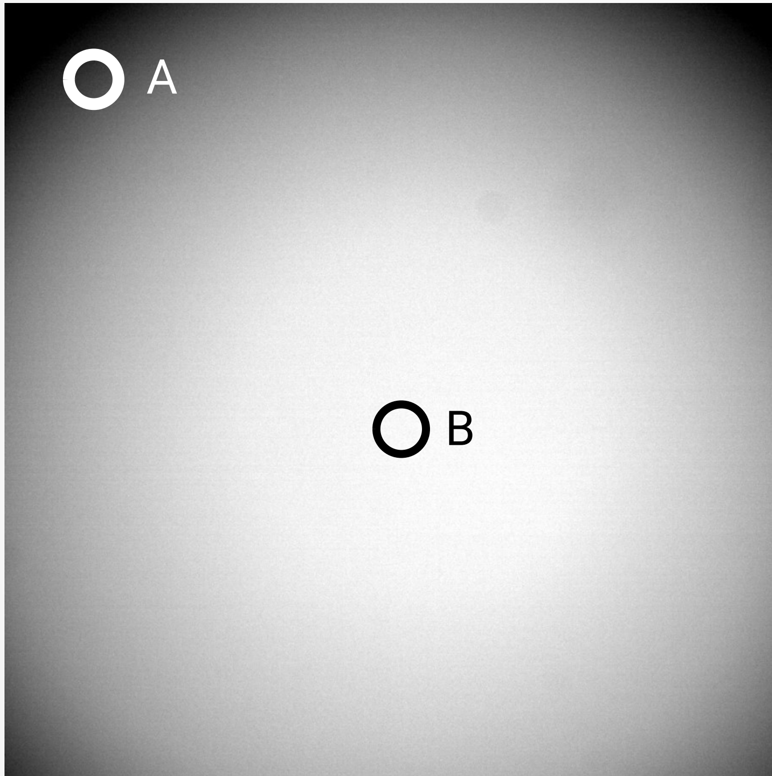


Image captured on a
widefield microscope, 10x objective

Assume you have the same fluorescent object. At which spot will that object appear brighter?

A or B

What is the effect of uneven illumination?



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widefield microscope, 10x objective

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A or B

Why is this a problem for intensity measurements?

What is the effect of uneven illumination?

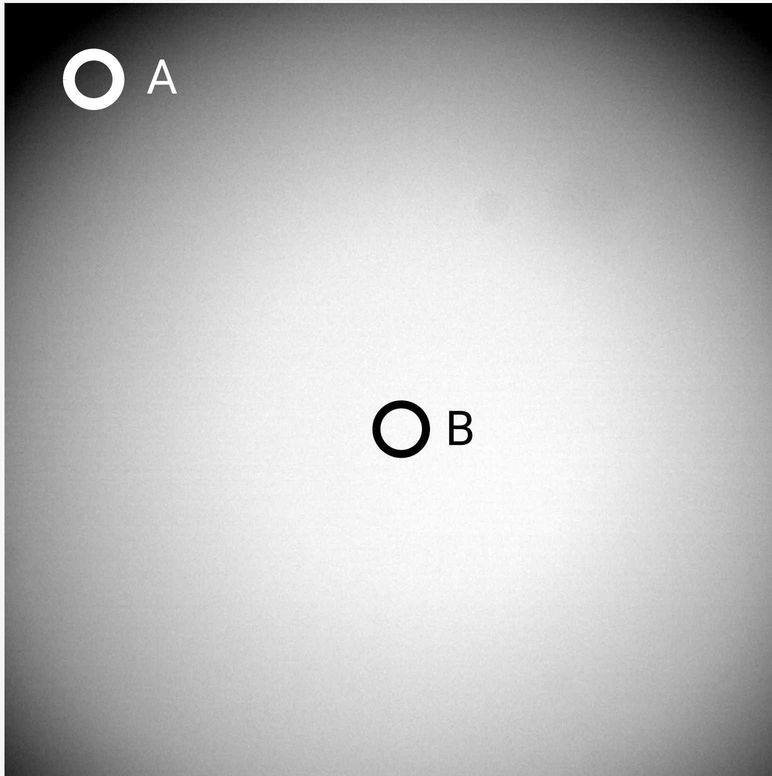

Image captured on a
widefield microscope, 10x objective

Assume you have the same fluorescent object. At which spot will that object appear brighter?

A or B

Why is this a problem for intensity measurements?

Typically we want intensity to correlate with cell function. This effect confounds our measurements.

How do we fix this issue?

- Remember that emission intensity is proportional to excitation intensity

- To correct for uneven illumination we simply divide the image by the illumination calibration image

Task

- Download the images kidney.tif and illumination.tif
- Correct the uneven illumination pattern in the kidney.tif image

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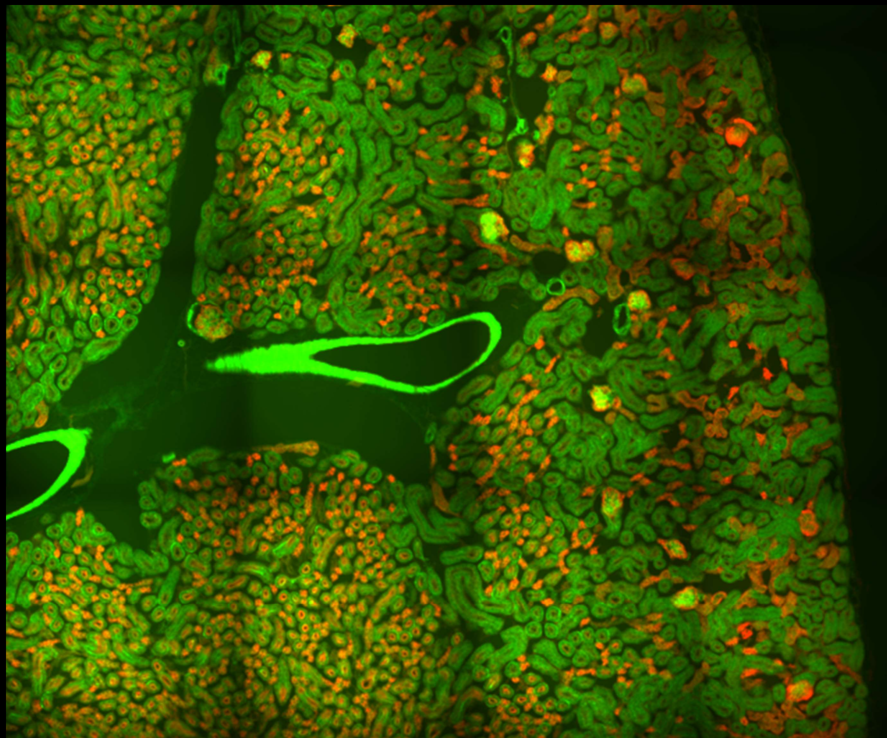
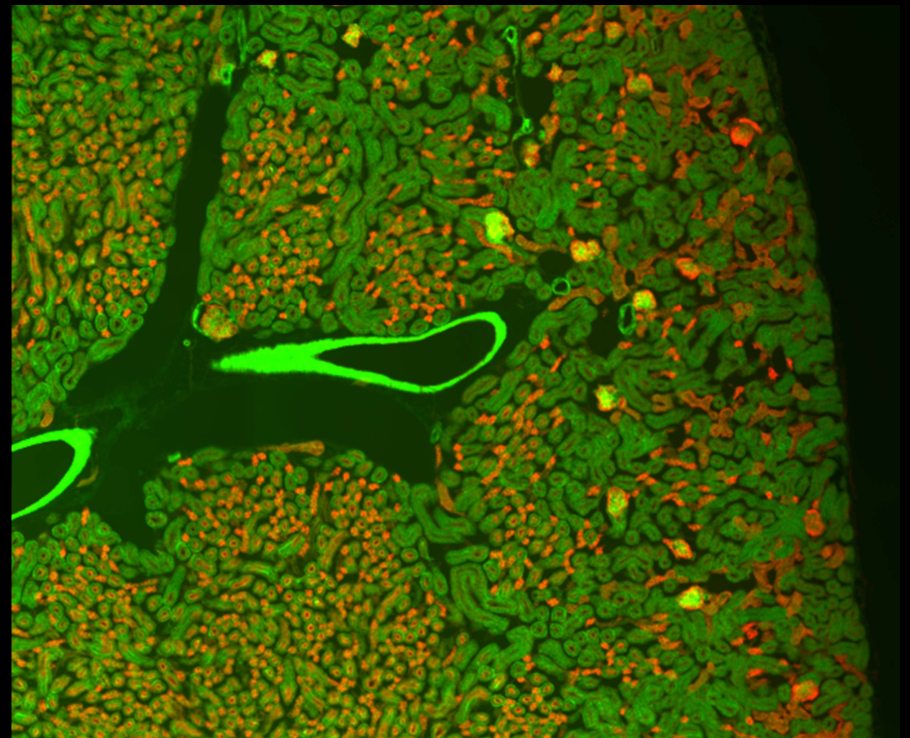
- Correct the uneven illumination pattern in the kidney.tif image

Don't forget to convert the unsigned integer images to doubles BEFORE dividing

e.g. `double(Ikidney)`



Uneven illumination



Corrected

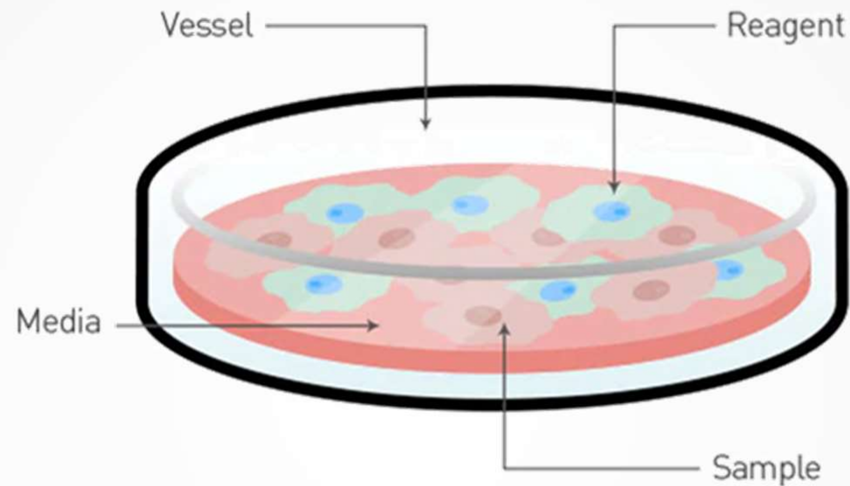
http://nic.ucsf.edu/blog/2014/01/shading-correction-of-fluorescence-images/

Background fluorescence

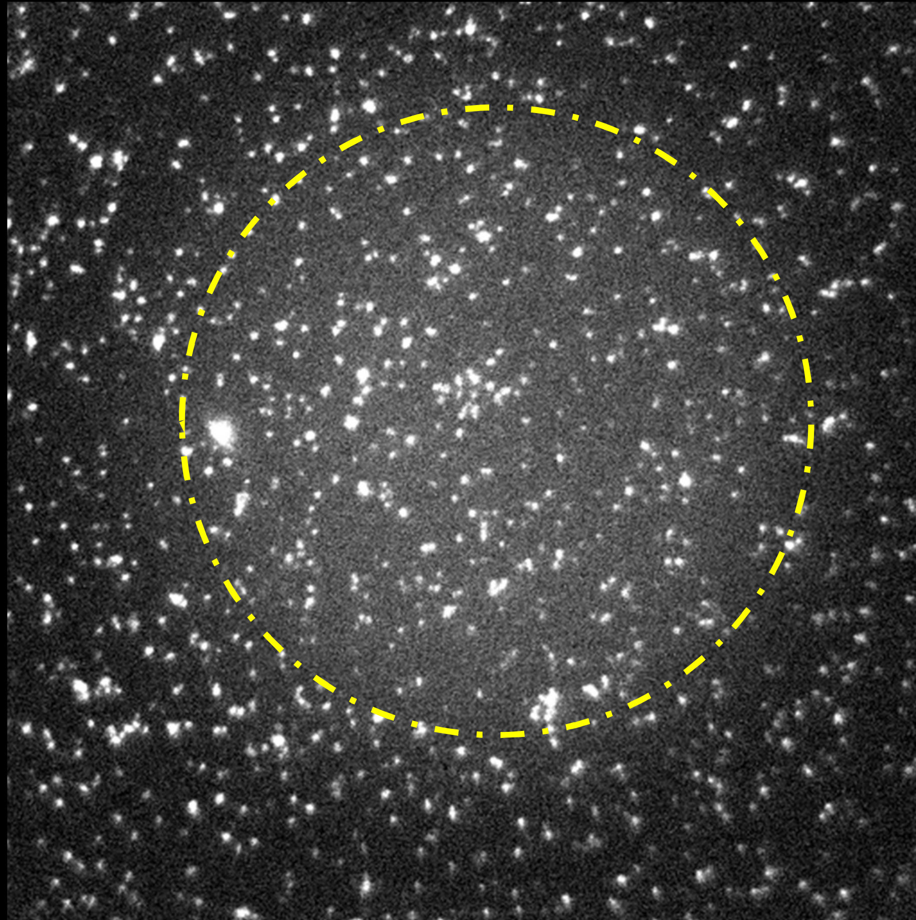
What is background fluorescence?

Background fluorescence (or noise) is signal that you can see but you don't want

Sources of background fluorescence

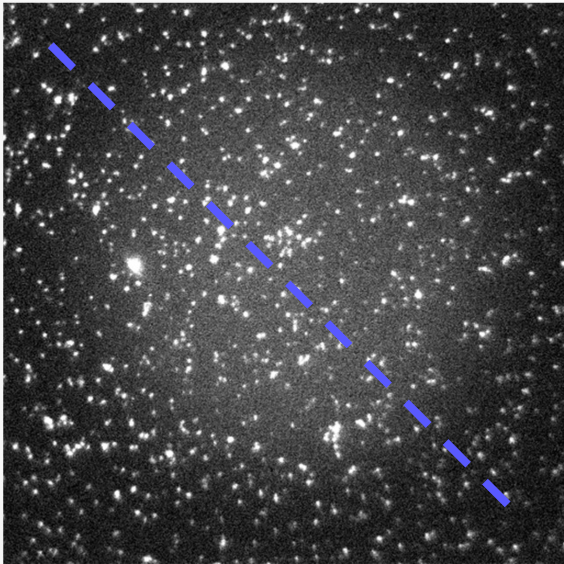


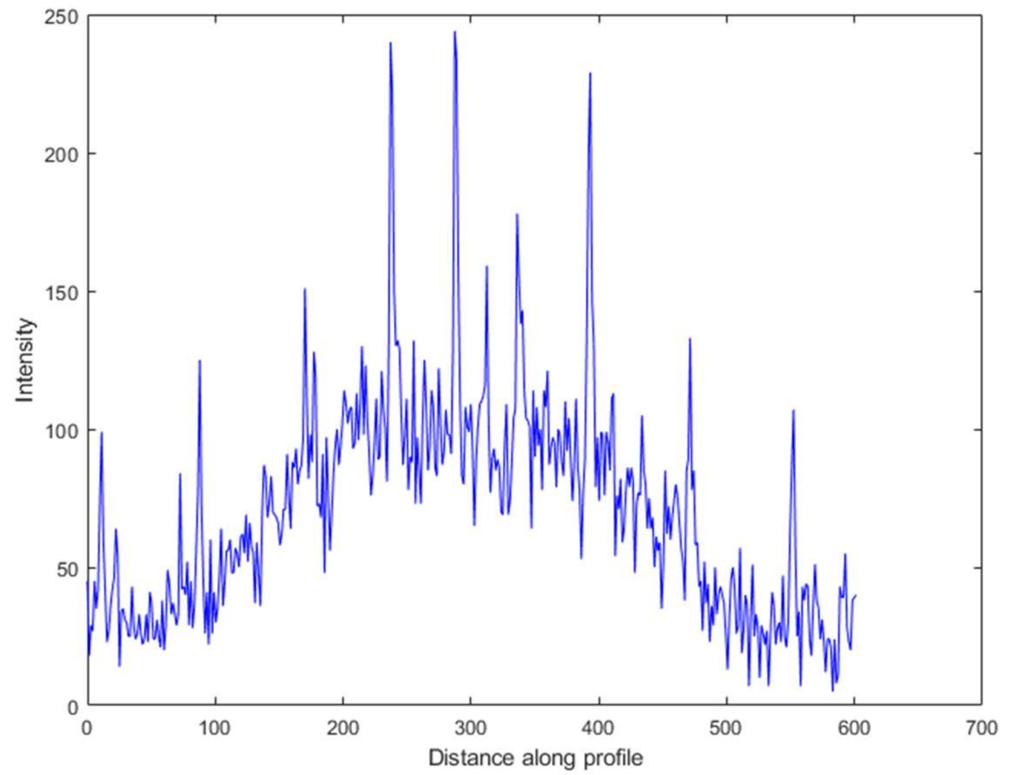
- Sources:
  - Due to instrument setup and imaging parameters (e.g. light from excitation leaking through, camera noise, ambient light)
  - Autofluorescence from samples, vessels, growth media, or unbound fluorophores



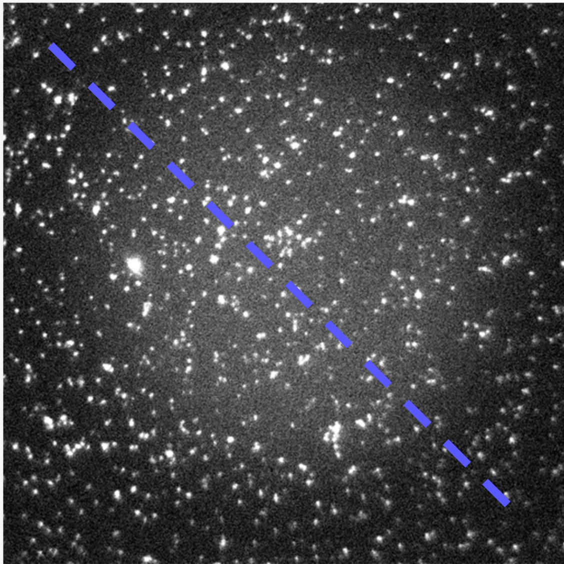
Background due to medium autofluorescence

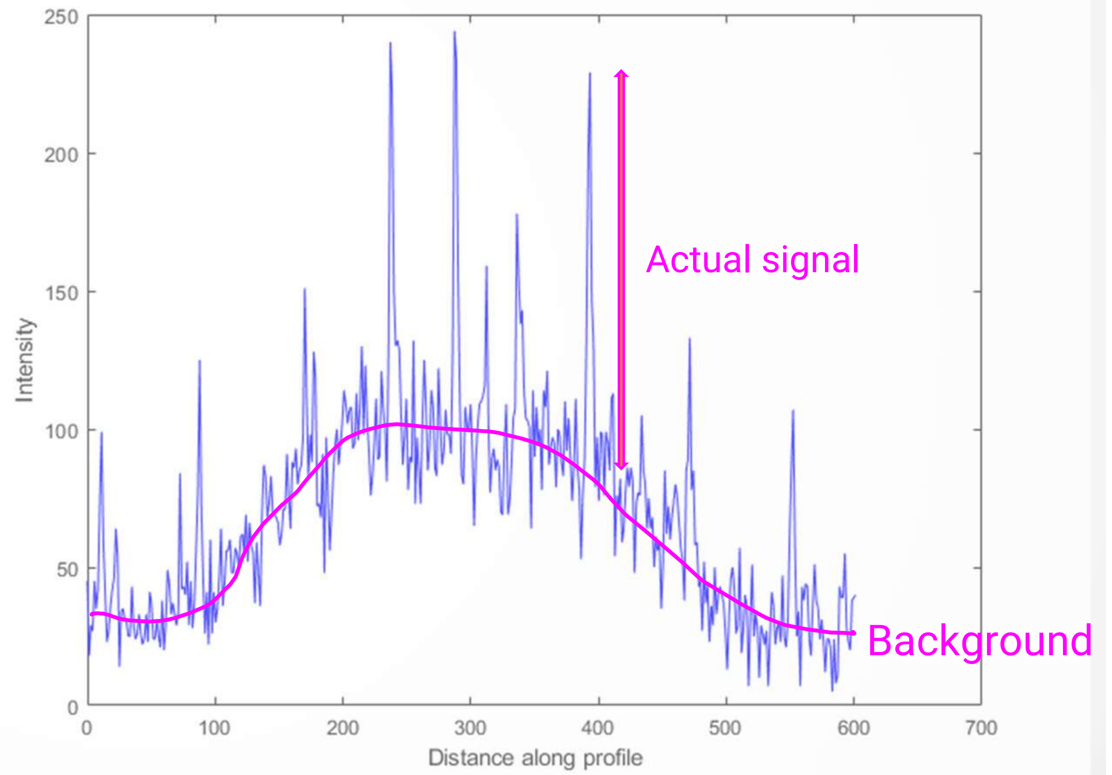
http://help.imageanalyst.net/workflow_BackgroundSubtraction.html





Intensity
Distance along profile
250
200
150
100
50
0
0
100
200
300
400
500
600
700



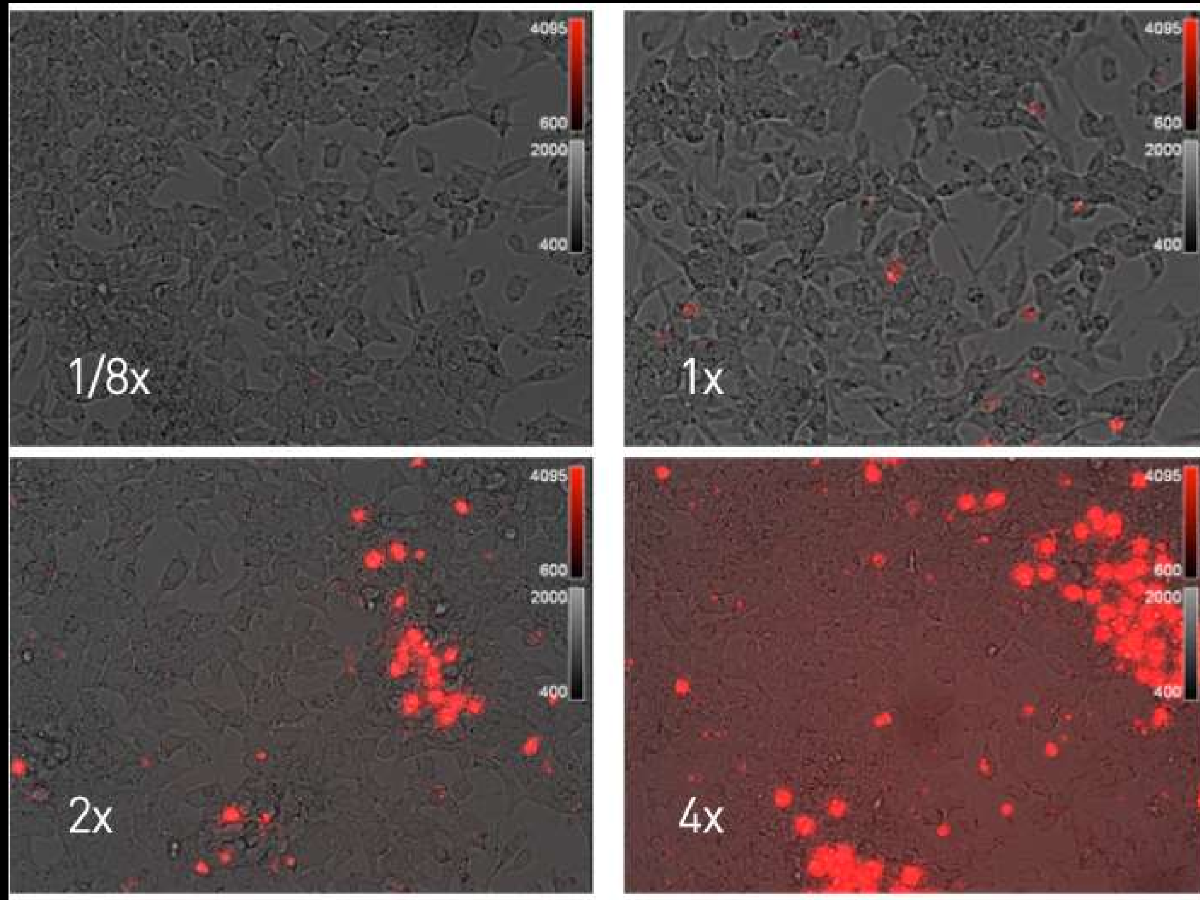


Actual signal
Background
Intensity
Distance along profile
0
50
100
150
200
250
0
100
200
300
400
500
600
700

How to deal with background fluorescence?

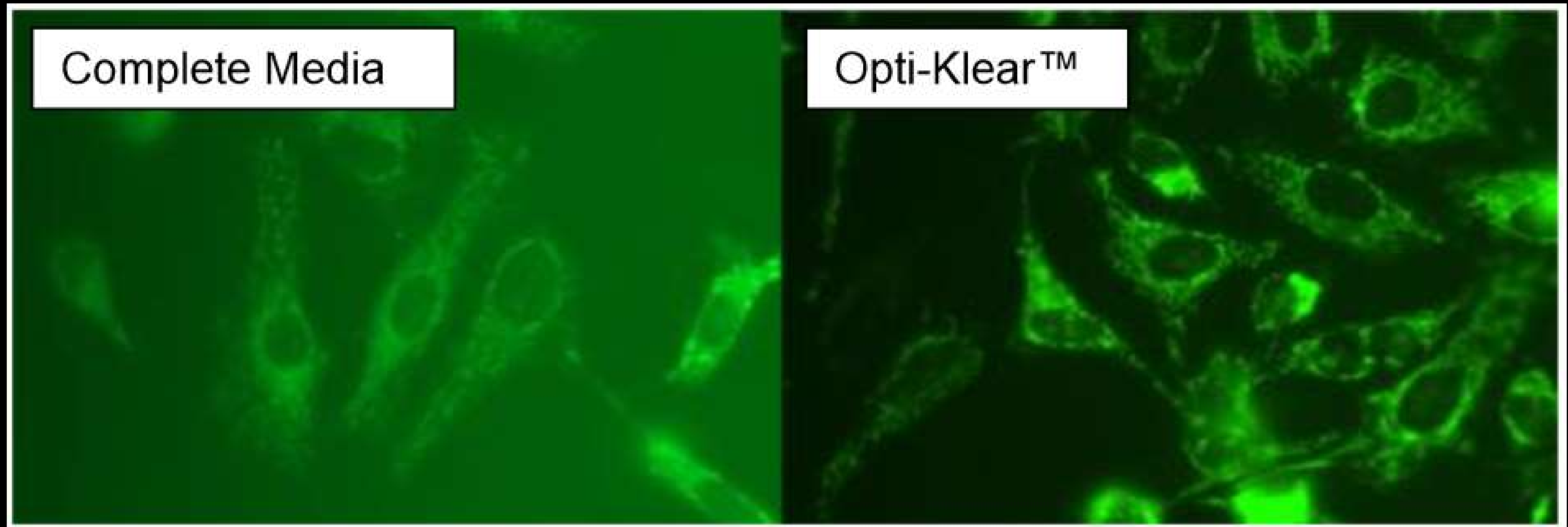
- Deal with imaging/experimental issues first
  - Turn off the room lights
  - Wash samples after labeling
  - Optimize amount of dye used
  - Try changing dyes for a different color
  - Change background media

Different dye concentrations



Thermo Fisher

Change background media



Often proteins and vitamins will fluoresce

Image stolen from the internet

How to deal with background fluorescence?

- Deal with imaging/experimental issues first
  - Turn off the room lights
  - Wash samples after labeling
  - Optimize amount of dye used
  - Try changing dyes for a different color
  - Change your background media

- If all else fails, then correct computationally

Background fluorescence adds to image intensity

$$I = I_{sample} + I_{background}$$

So to correct, you subtract the background

Two ways to get a background image

- Best way: Capture a background image of the autofluorescence

- Estimate background fluorescence:
  - Greyscale morphological opening
  - Median filtering
  - … many other ways!

Capture an image with media but no cells

Credit: Adrian Ramirez (Liu lab)

Estimating the background

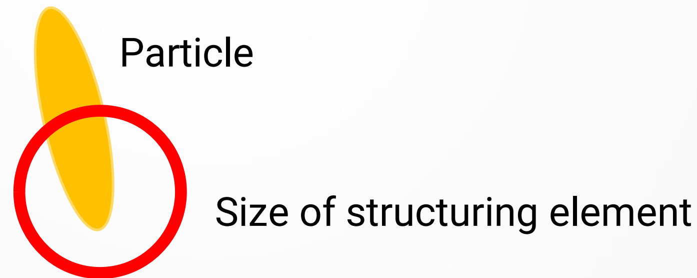
- To estimate the background, use grayscale morphological opening

- 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

Estimating the background

- 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



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 (optional)
4. Subtract the background

`B = imgaussfilt(A, sigma)`

Smooths/blurs image A with a Gaussian of width sigma.

The higher the sigma value, the blurrier the image appears

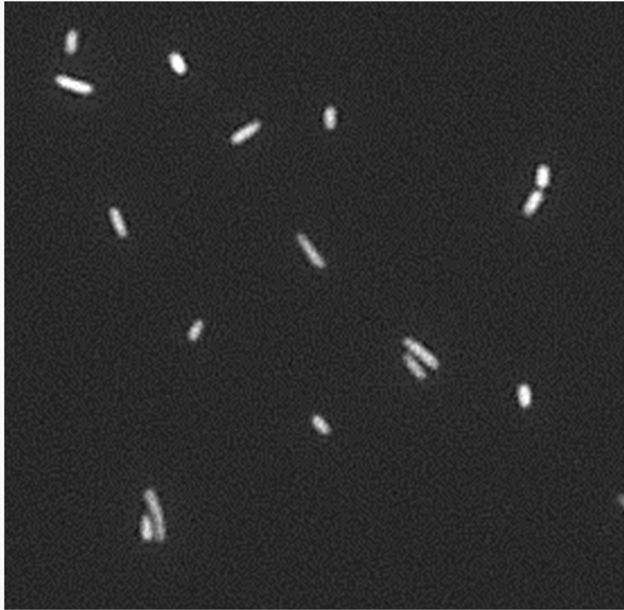
MATLAB code

```
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, [])
```

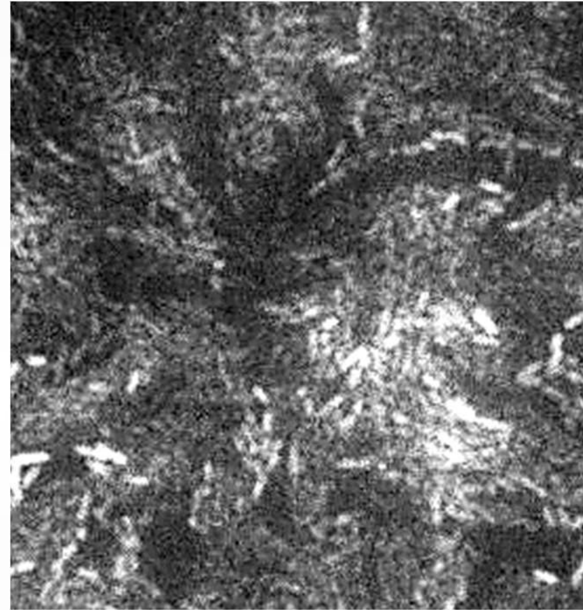
Estimating the background

- This approach only works when there are large regions of background
  - Image cannot be crowded with cells
  - The cells are not in large colonies

Suitable images for background estimation



Good image
- Cells are well separated
- Plenty of background pixels to sample



Bad image
- There are too many cells
- No background pixels
- Estimation will not work

Summary

- To correct for uneven illumination, divide the image by the illumination calibration image
- To correct for background fluorescence, subtract the image by the background image
- Remember to convert the images to double **before** doing these operations
- Ideally, acquire both calibration images for every experiment

Full correction

$$I_{corr} = \frac{I - I_{bg}}{I_{illum}}$$