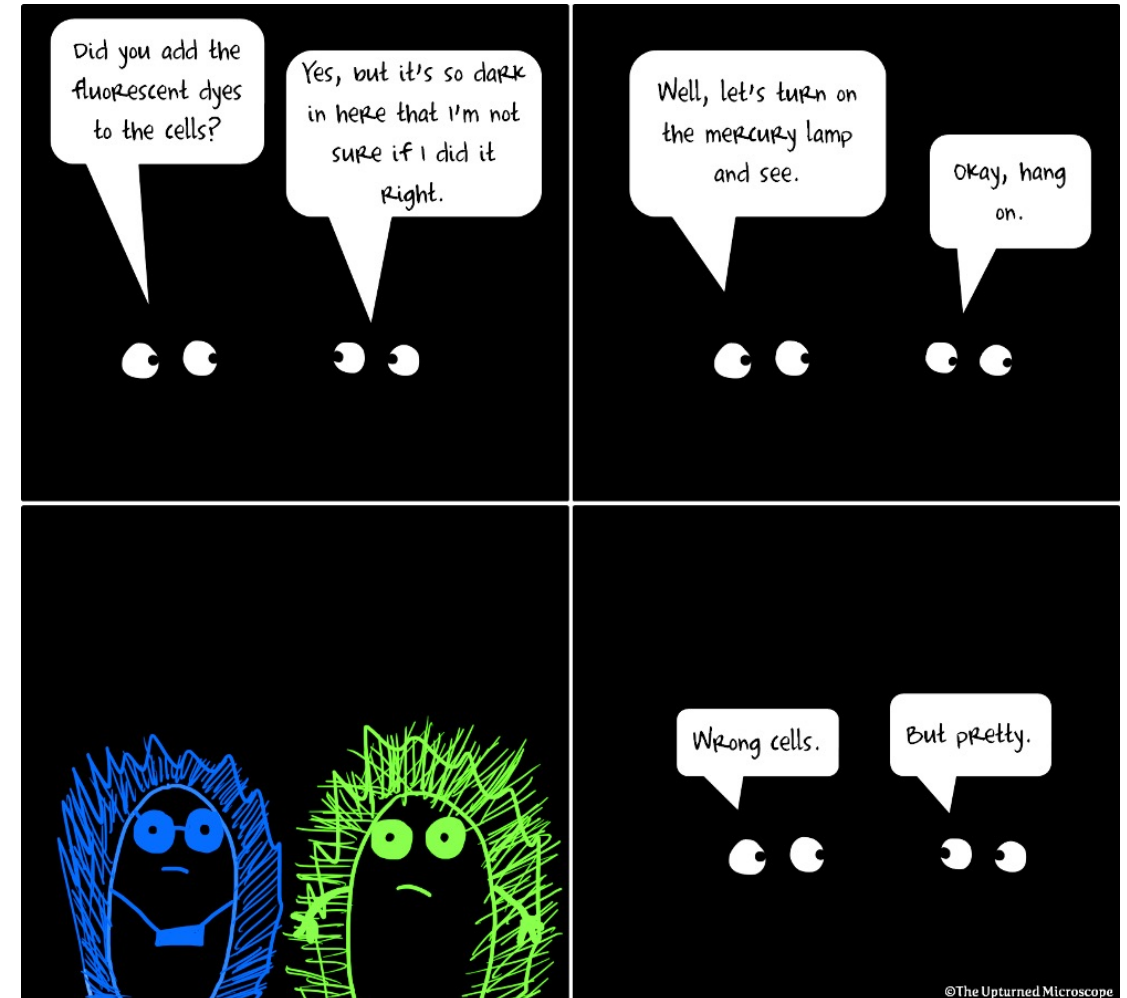
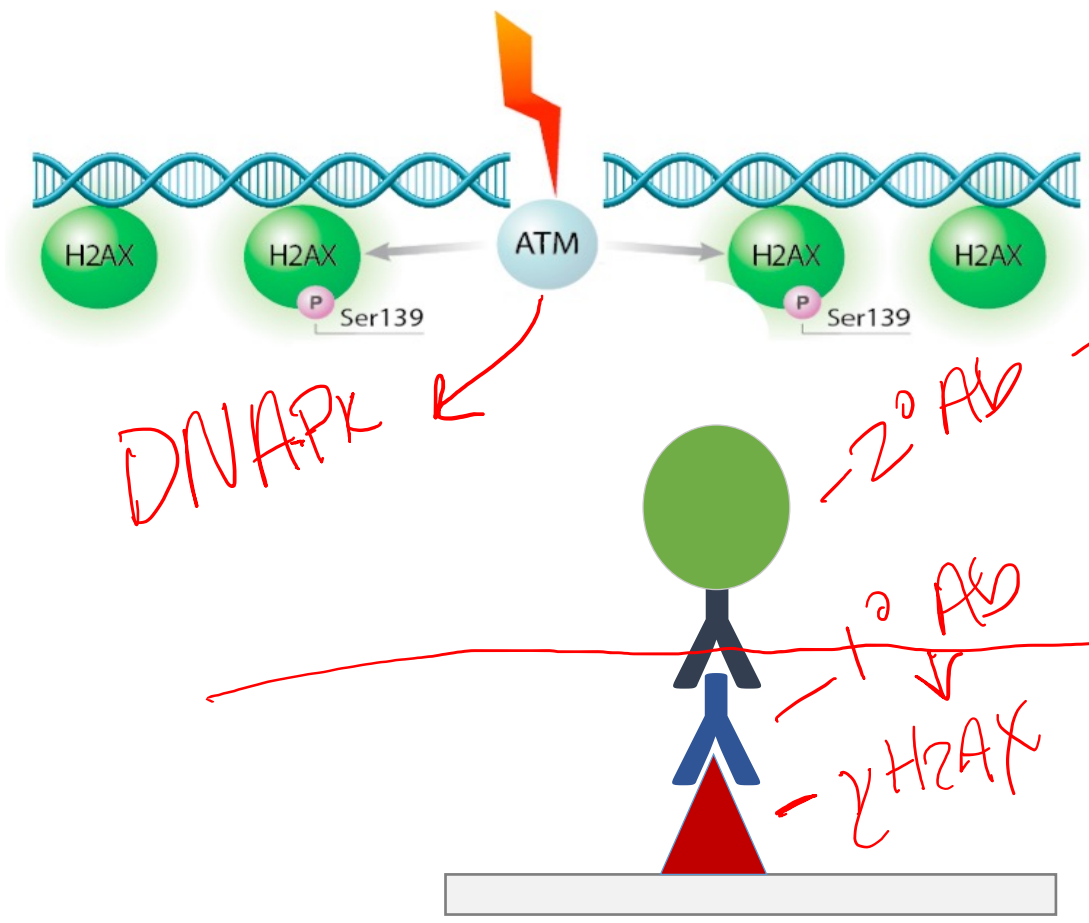


# M1D3: Use immunofluorescence staining to assess repair foci experiment

- ✓ 1. Comm Lab
2. Prelab
3. Antibody staining for  $\gamma$ H2AX assay
4. Image analysis







# Using immunofluorescence: $\gamma$ H2AX assay to detect double-strand DNA breaks



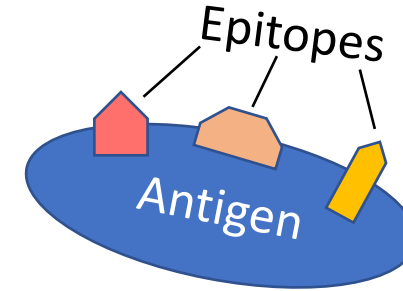
- Histone H2AX phosphorylated at Ser139 if DSB
- Antibodies against  $\gamma$ H2AX (phosphorylated form)

*recognizes 1<sup>st</sup> Ab*  
*tagged for visualization*

|   |  |
|---|--|
| protein of interest   |  $\gamma$ H2AX                        |
| primary antibody  |  mouse anti-human anti- $\gamma$ H2AX |
| secondary antibody  |  goat anti-mouse                    |
| Fluorophore (conjugated to secondary antibody)<br>exc./ em. wavelengths |  488/525 nm                         |

# Considerations for using antibodies in the lab

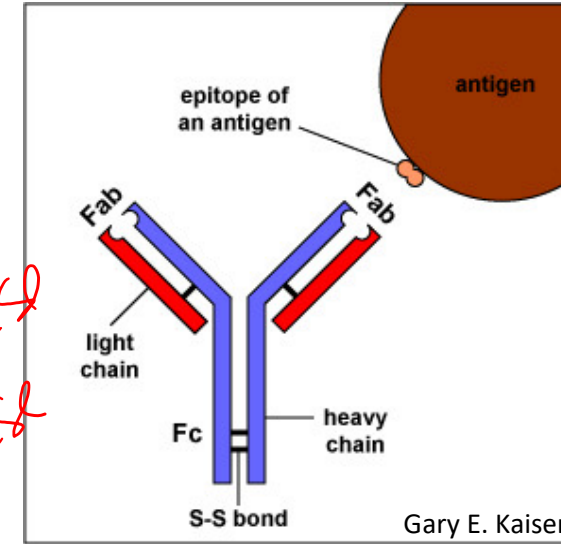
- Antibodies bind to specific epitopes on antigens
  - Antigens may have multiple epitopes



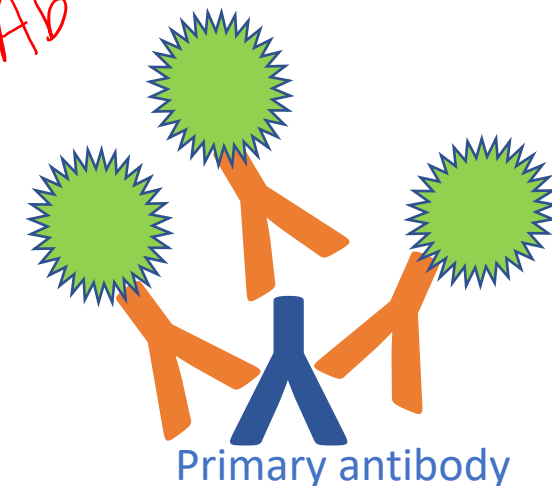
## Primary antibodies vs secondary antibodies

- ~~Primary~~ antibody recognizes the antigen
  - Specific protein sequence
  - Specific conformation of protein
  - Specific state of protein (i.e. phosphorylation)

Highly specific  
10 Ab = mouse  
20 Ab = α mouse

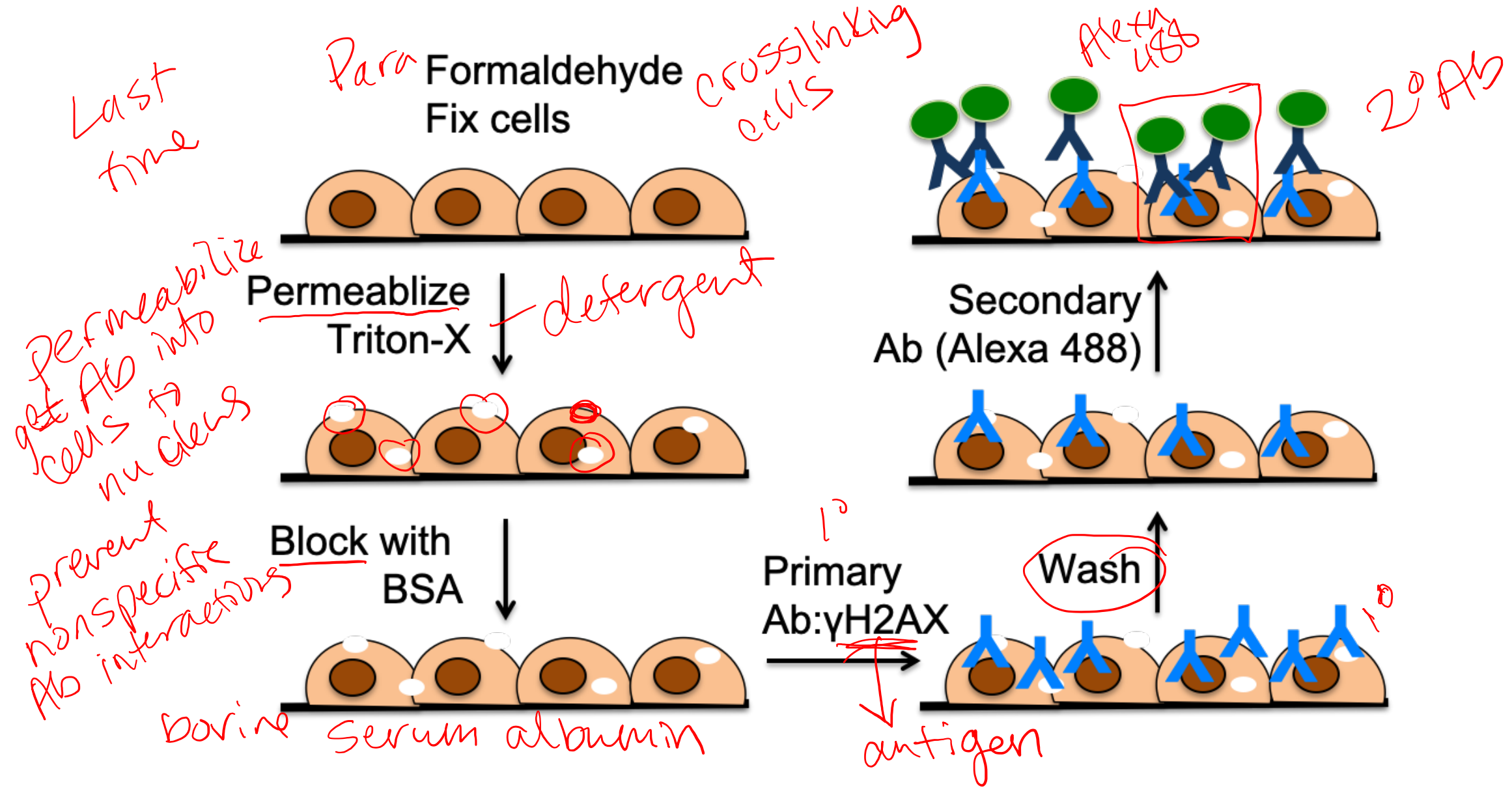


- ~~Secondary~~ Ab recognizes the species of the primary Ab
  - Often conjugated to tag for visualization
    - Enzyme or fluorophore
  - Amplifies signal through multiple bindings
  - Consider sample species when choosing antibodies!

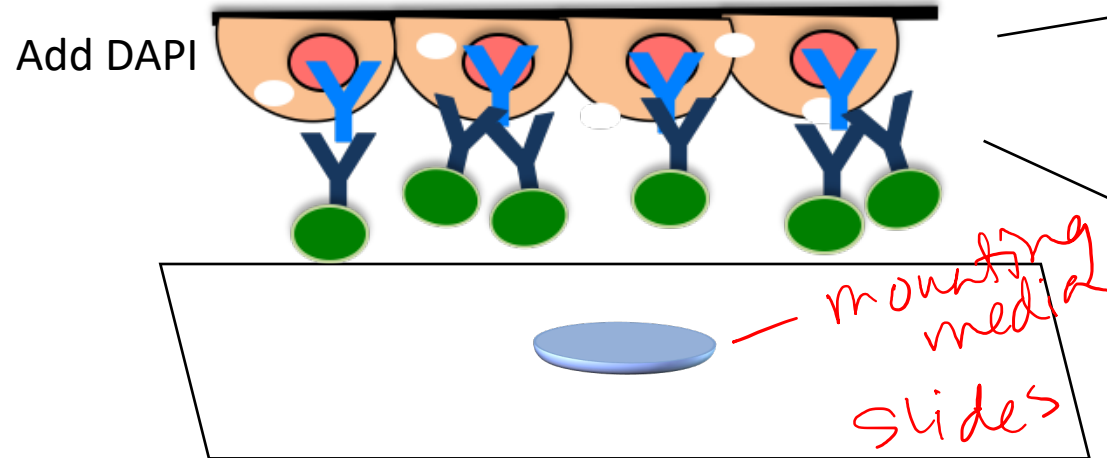


Secondary antibody conjugated to a fluorophore

# Using immunofluorescence (IF): steps in protocol

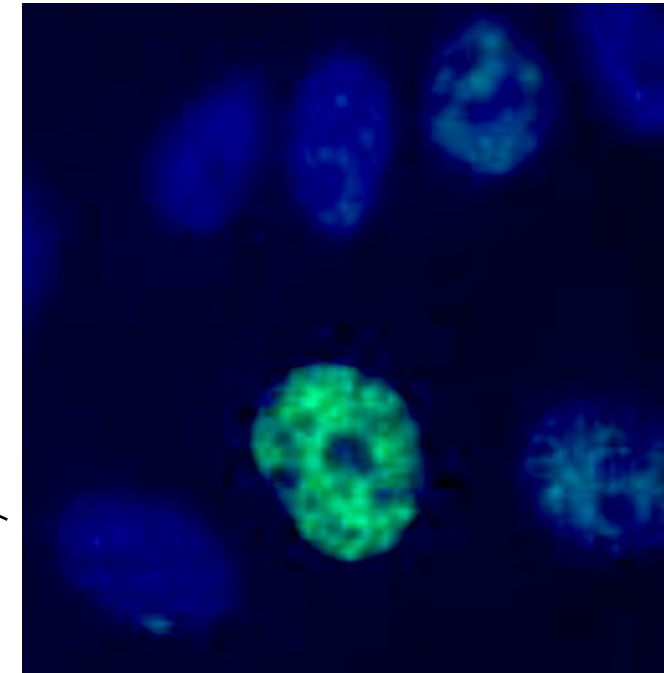


# Finish IF by adding DAPI, then mount slides for imaging



Mount coverslip on glass slide  
with mounting media

flip  
CS



DNA stain / dye  
1  
Blue = DAPI  
Green =  $\gamma$ H2AX

# How will we analyze our images?

## ImageJ

- Open source software developed at the NIH and used around the world



# ImageJ

Image Processing & Analysis in Java

## Two types of ImageJ analysis

### 1. Intensity

- Measure average FITC signal intensity per nuclei

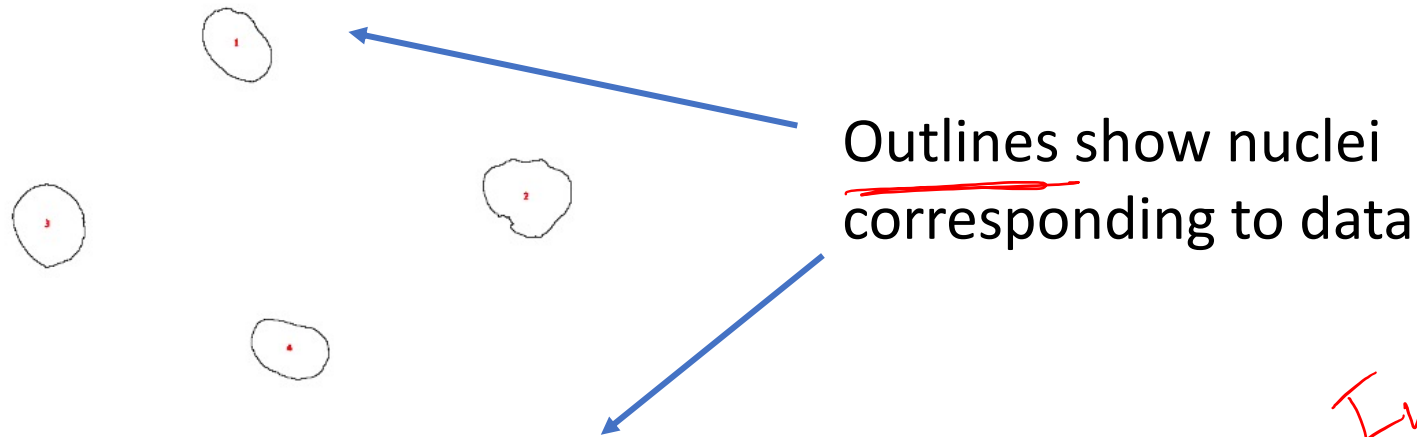
### 2. Foci counting

- Measure average FITC punctae number per nuclei

- 4 conditions  
- 8 images  
    ↳ multiple nuclei  
→ consider each  
    image =  $n$   
     $n = 8$

# Perform ImageJ analyses for fluorescent intensity and foci counting

- Set threshold and mask to only examine FITC signal within DAPI-stained nuclei
- Results should be copied into Excel sheet for analysis



| Results |                             |      |        |     |     |       |        |
|---------|-----------------------------|------|--------|-----|-----|-------|--------|
|         | Label                       | Area | Mean   | Min | Max | Circ. | IntDen |
| 1       | 082620_MCL5_notx_01007-0002 | 4291 | 11.407 | 10  | 14  | 0.829 | 48949  |
| 2       | 082620_MCL5_notx_01007-0002 | 5995 | 15.549 | 10  | 42  | 0.787 | 93215  |
| 3       | 082620_MCL5_notx_01007-0002 | 5316 | 20.894 | 12  | 53  | 0.868 | 111072 |
| 4       | 082620_MCL5_notx_01007-0002 | 4213 | 10.755 | 9   | 17  | 0.831 | 45309  |

- Intensity  
code  
→ save into  
excel (copy)



# H2AX analysis

AKA: What am I supposed to do with all these numbers?!

*nuclear area*

| Results |                             |      |        |     |     |       |        |           |
|---------|-----------------------------|------|--------|-----|-----|-------|--------|-----------|
|         | Label                       | Area | Mean   | Min | Max | Circ. | IntDen | RawIntDen |
| 1       | 082620_MCL5_notx_01007-0002 | 4291 | 11.407 | 10  | 14  | 0.829 | 48949  | 48949     |
| 2       | 082620_MCL5_notx_01007-0002 | 5995 | 15.549 | 10  | 42  | 0.787 | 93215  | 93215     |
| 3       | 082620_MCL5_notx_01007-0002 | 5316 | 20.894 | 12  | 53  | 0.868 | 111072 | 111072    |
| 4       | 082620_MCL5_notx_01007-0002 | 4213 | 10.755 | 9   | 17  | 0.831 | 45309  | 45309     |
| 5       | 082620_MCL5_notx_01007-0002 | 5888 | 18.811 | 11  | 46  | 0.261 | 110757 | 110757    |

File Name

area of region measured (px<sup>2</sup>)

sum of gray values/number of pixels

ranges from 0-1  
1= perfect circle

sum of value of pixels in region measured



# In lab today:

1. Complete IF staining for H2AX
2. View H2AX images on 7<sup>th</sup> floor microscope
3. Analyze H2AX images with batch analysis

## HW due M1D4

(Biorender)

(group)

- Create an experimental schematic for the H2AX staining process

(individual)

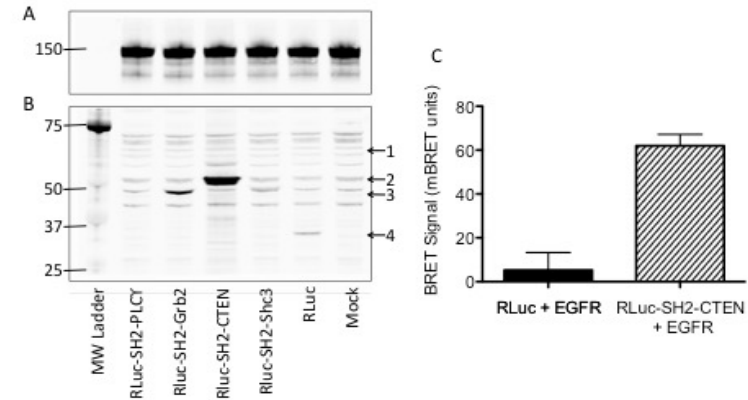
1. Create a data figure of H2AX results with title and caption
2. Visit Comm Lab before M1D5.

# Results slide example

for 1/2  
1/3 page

- Image **should not** be the entire page
  - Only needs to be large enough to be clear / visible
- Title **should** be conclusive
  - Don't include what you did, rather state what you found (take home message)
- Caption **should not** detail the methods or interpret the data
  - Define abbreviations, symbols, etc.
  - Info needed to "read" figure
- Bullet points **should** present and interpret the data

Data  
Summary  
figure



**Figure 1: Development of BRET assay to monitor EGFR and SH2 domain interactions.** CHO-K1 cells were transfected with Citrine-EGFR (A) and renilla luciferase (RLuc)-tagged SH2 domains from PLCg, Grb2, CTEN, and Shc3 (B). Western blots of CHO-K1 lysates were probed with anti-EGFR (A) or anti-RLuc (B) antibodies. Arrowheads indicate the expected molecular weight of the RLuc-tagged proteins; (1) RLuc-SH2-PLCg, (2) RLuc-SH2-CTEN, (3) RLuc-SH2-Grb2 and RLuc-SH2-Shc3, and (4) RLuc alone. Mock indicates no cDNA was utilized during transfection. (C) For CTEN only, BRET signal was quantified using a luminometer after stimulation of CHO-K1 with 100 ng/mL EGF for 15 min.

## **BRET system effectively measures EGFR activation:**

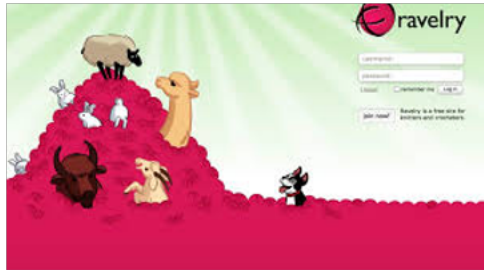
- To determine if the BRET system could be used to monitor EGFR activation, CHO-K1 cells were transfected with fluorescent EGFR and luciferase-tagged SH2 domains and a BRET assay was performed after growth factor stimulation.
- CHO-K1 were transfected with Citrine-EGFR in all conditions as indicated by correct molecular weight band at 150 kDa (Figure 1A).
- Several protein bands are present in Mock transfection lane suggesting off-target binding of the RLuc antibody (Figure 1B).
- RLuc alone, RLuc-SH2-Grb2, and RLuc-SH2-CTEN were successfully transfected as indicated by correct molecular weight bands (Figure 1B).
- RLuc-SH2-PLCg and RLuc-SH2-Shc3 did not appear by Western blot analysis -- bands different from those in the Mock lane are not identifiable. This outcome could be due to protein expression levels below the detection limit by Western blot or to unsuccessful transfection of cDNA.
- BRET signal increased in cells transfected with Citrine-EGFR and RLuc-SH2-CTEN versus Citrine-EGFR and RLuc alone after EGF stimulation. This difference suggests that the BRET signal is specific for an SH2-EGFR interaction versus randomly localized RLuc.
- In sum, these data suggest that the RLuc-SH2 constructs can be utilized to monitor EGFR phosphorylation, as SH2 domain-EGFR association occurs only at sites of EGFR tyrosine phosphorylation. Next, we determined the dynamic range of the BRET assay.

# Notes on experimental schematics...

How does Becky knit a scarf?



Buy beautiful yarn



Choose a pattern



Cast on 25 stitches



Knit



Purl

K & P & K & P

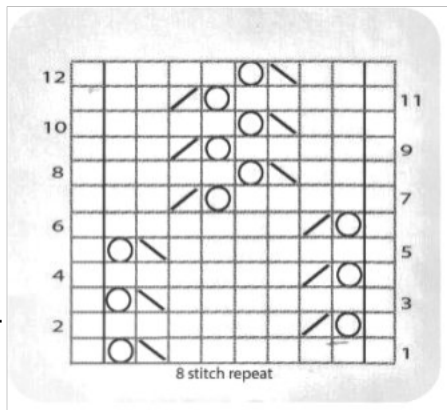
Measure size. It  
doesn't match  
the  
recommended  
gauge



Frog it!



Cast on 40 stitches



Follow the pattern until  
time to cast off



Block scarf to wear

# What should be in the Title and Caption?

**Title:** State what is shown / represented in the schematic

**Caption:**

- Explain the flow of information using concise / clear language
- Expand on text shown in figure labels to eliminate excess wordiness / clutter from the figure
- Define all abbreviations / jargon / labels / symbols



# Revised example:

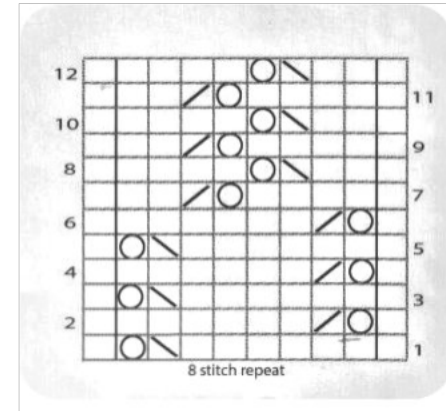
*Schematics  
DO NOT  
wiki  
slides*



Buy yarn and chose  
pattern



Swatch to check  
knitting gauge



Follow pattern repeats:  
K2,P2,YO,K2tog



Block for finished  
project

*X*

*X*

**Figure 1: Becky's knitting process.** Becky follows a specific protocol to knit a scarf. She choses her yarn and checks the pattern before following the written pattern and blocking to complete the project. K2= knit two, P2= purl 2, YO= yarn over, K2tog= knit two together