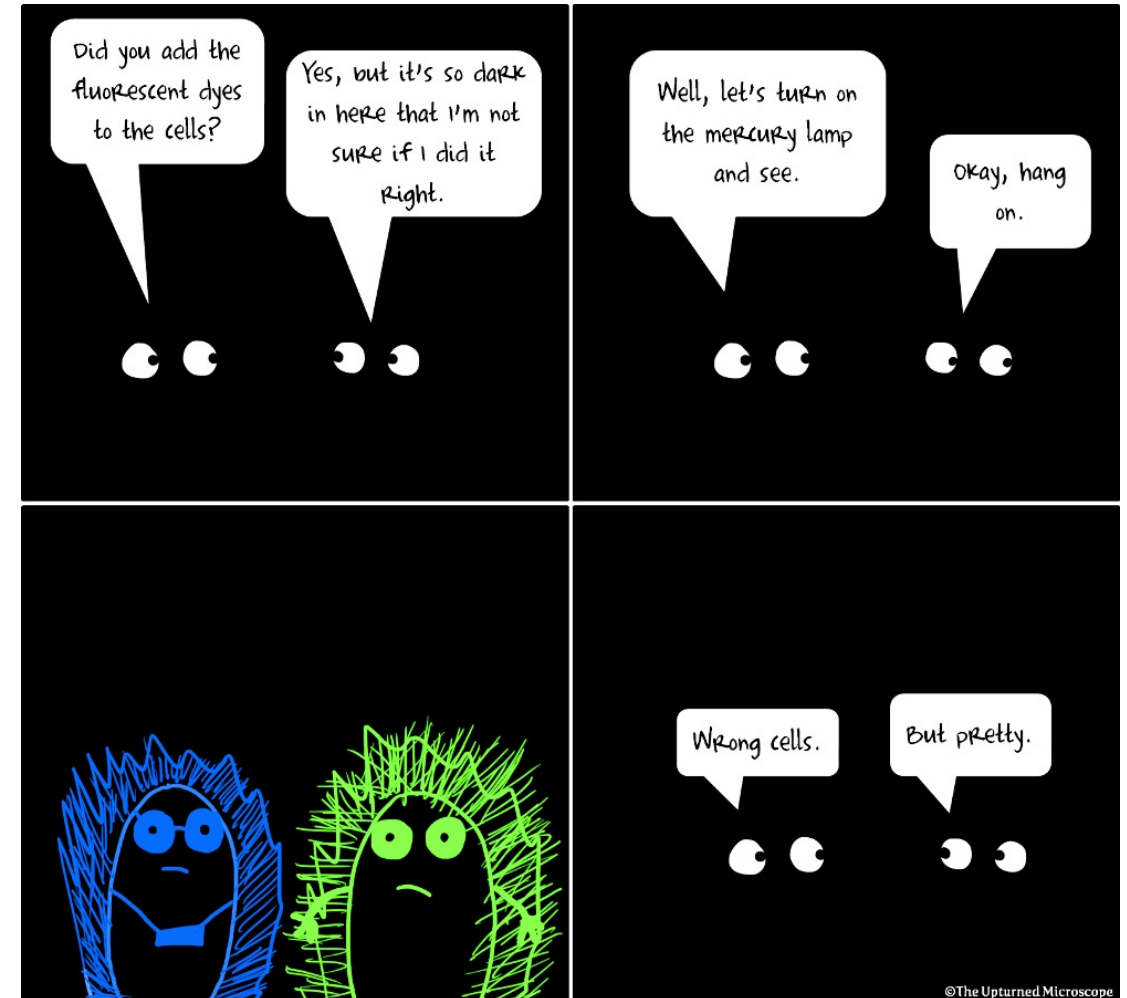
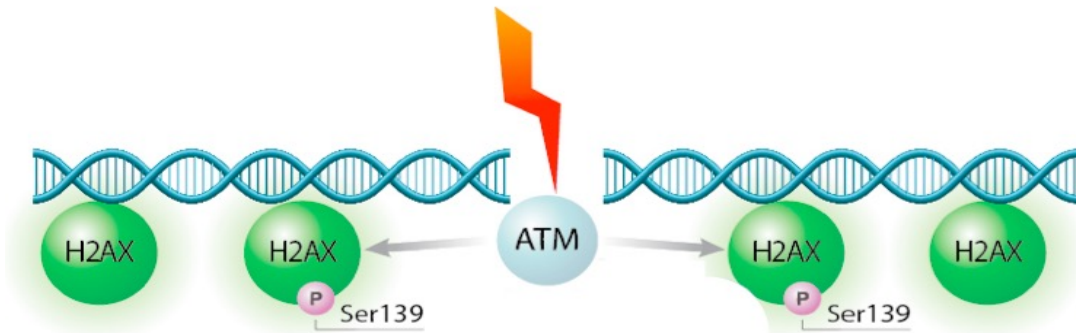


M1D3: Use immunofluorescence staining to assess repair foci experiment

1. Comm Lab
2. Prelab
3. Antibody staining for γ H2AX assay
4. Image analysis



Using immunofluorescence: γ H2AX assay to detect double-strand DNA breaks

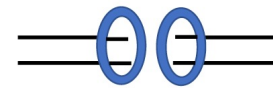


- Histone H2AX phosphorylated at Ser139 if DSB
- Antibodies against γ H2AX (phosphorylated form)

From lecture:

To join two
ends together,
you need
DNAPK

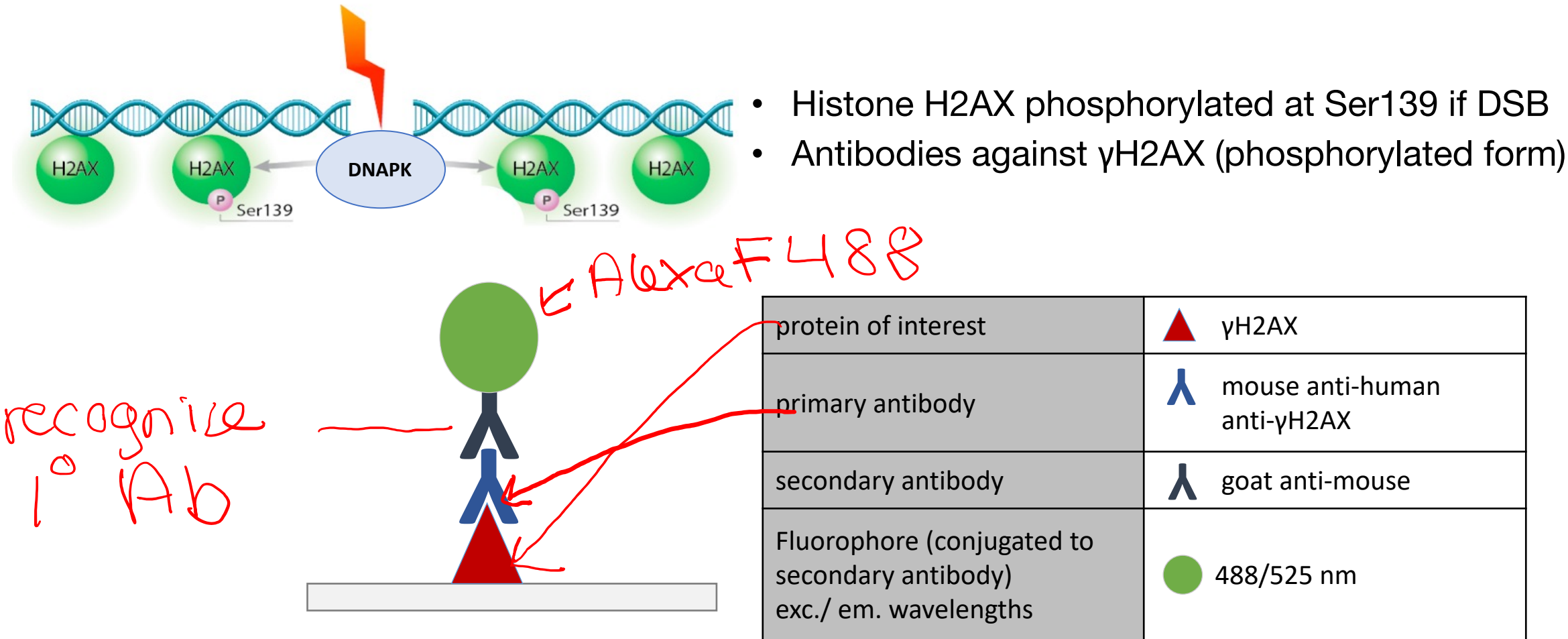
Ku70/80 Bind the Ends



DNAPK
Helps to
Bring the
Ends Together

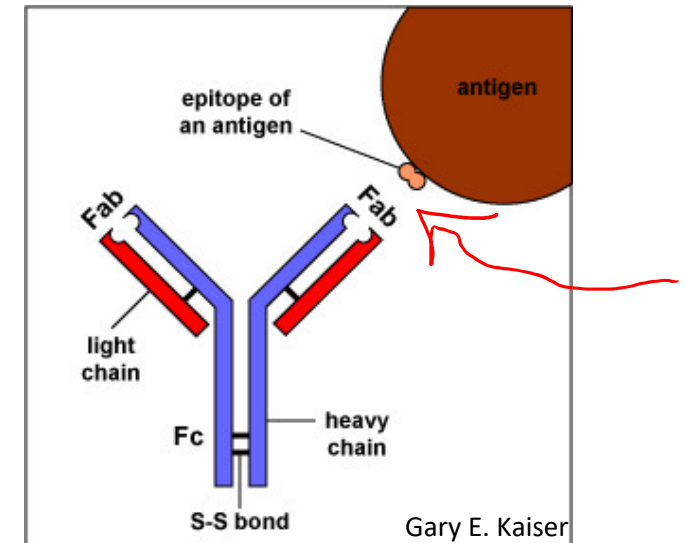
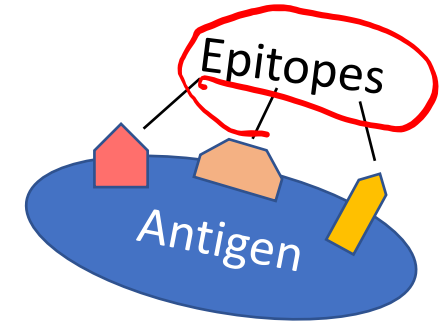
DNAPK is also a “DNA dependent protein kinase”
It phosphorylates many targets, including H2AX

Using immunofluorescence: γ H2AX assay to detect double-strand DNA breaks



Considerations for using antibodies in the lab

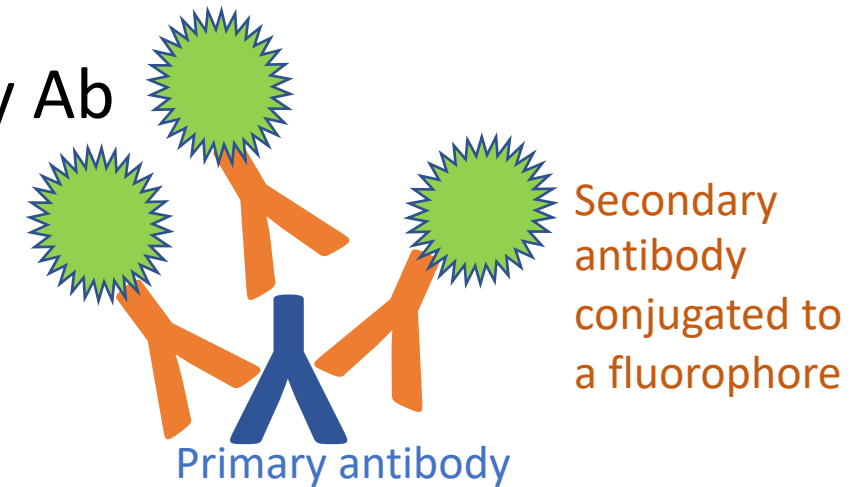
- Antibodies bind to specific epitopes on antigens
 - Antigens may have multiple epitopes
- Primary antibody recognizes the antigen
 - Specific protein sequence
 - Specific conformation of protein
 - Specific state of protein (i.e. phosphorylation)



Considerations for using antibodies in the lab

Primary antibodies vs secondary antibodies

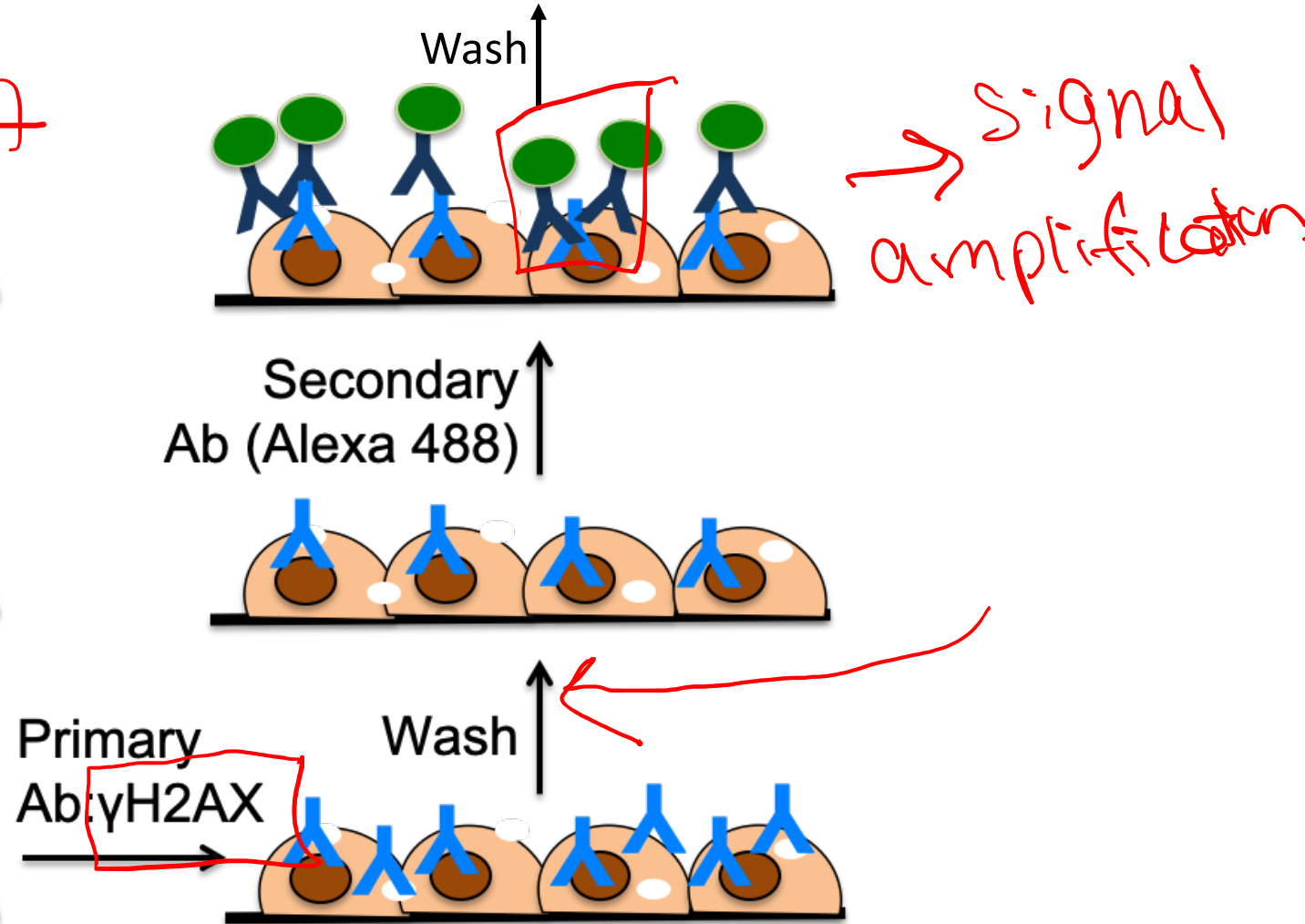
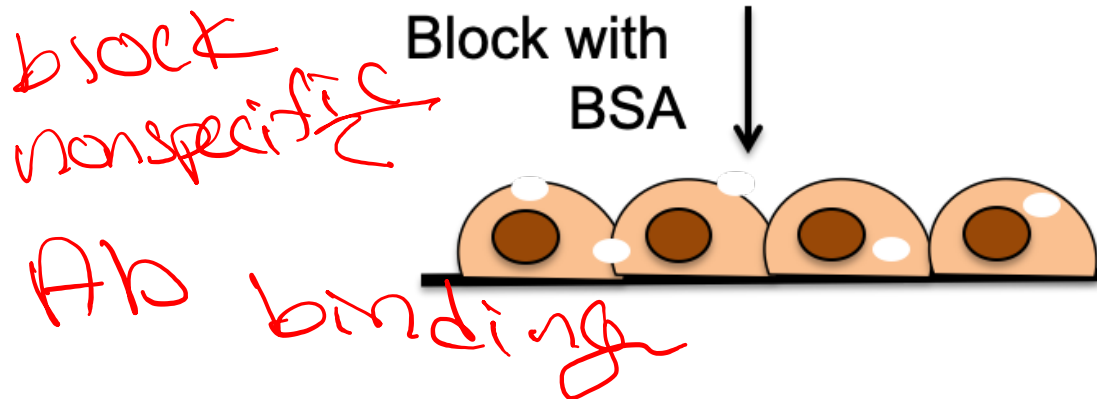
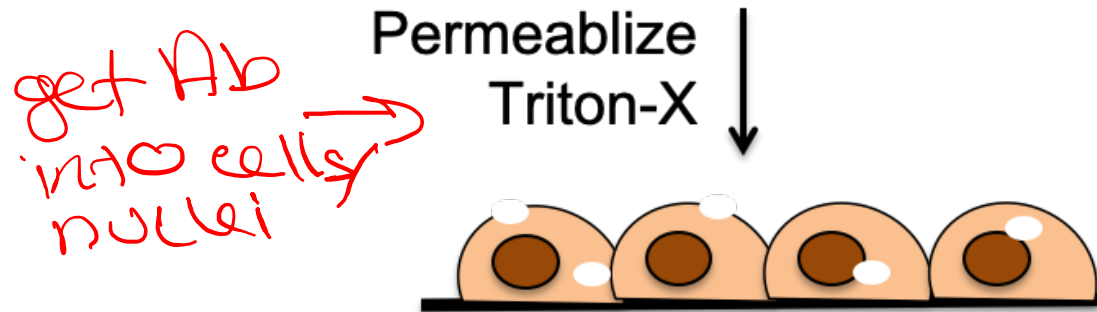
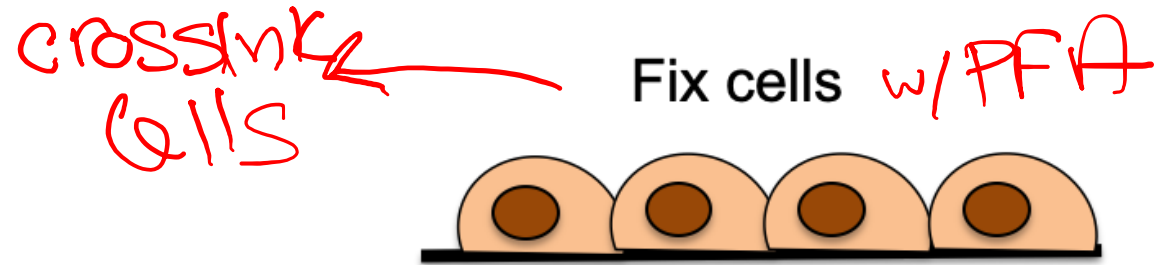
- Primary antibody recognizes the antigen
- Secondary Ab recognizes the species of the primary Ab
 - Often conjugated to tag for visualization
 - Enzyme or fluorophore
 - Amplifies signal through multiple bindings
 - Consider sample species when choosing antibodies!



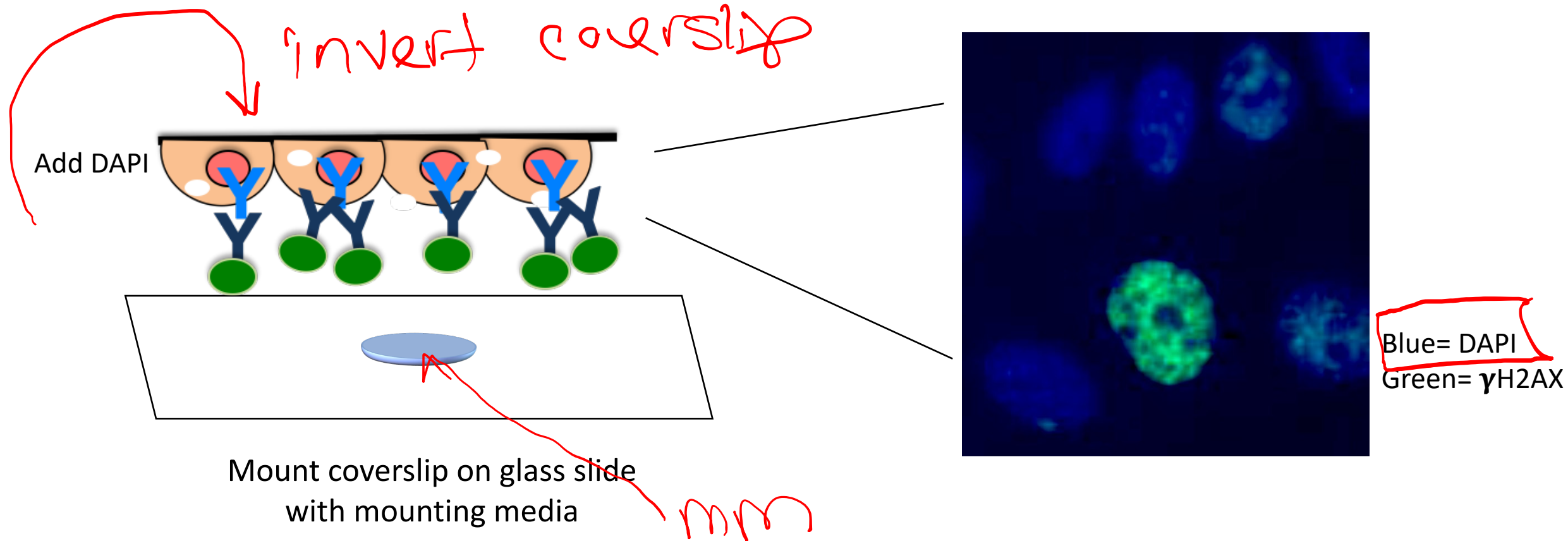
primary Ab: mouse

secondary Ab: α mouse

Using immunofluorescence (IF): steps in protocol



Finish IF by adding DAPI, then mount slides for imaging



DAPI = DNA binding dye

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

↑ yH2AX green

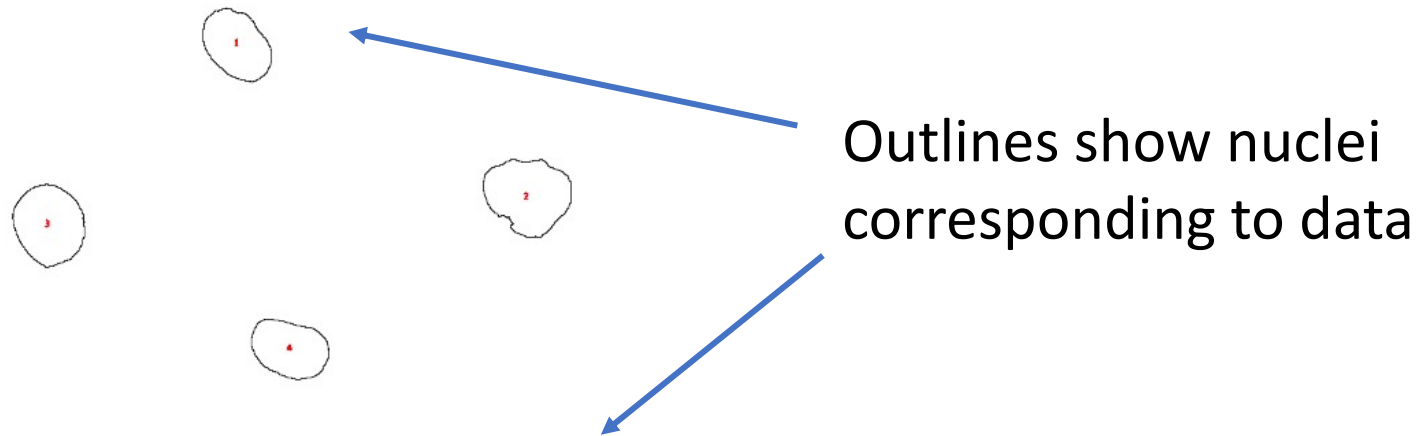
2. Foci counting

- Measure average FITC punctae number per nuclei

*4 groups
→ 8 images each
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

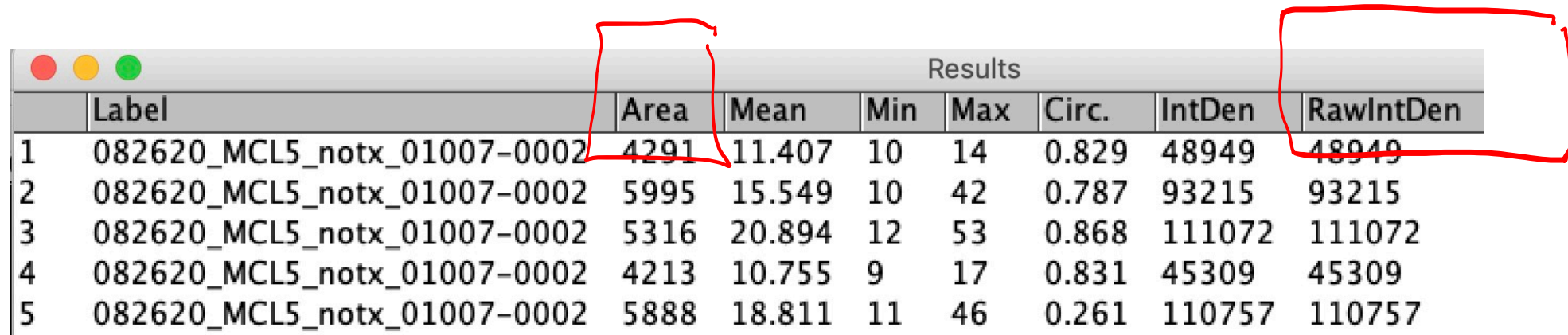


Results							
	Label	Area	Mean	Min	Max	Circ.	IntDen
1	082620_MCL5_notx_01007-0002	4291	11.407	10	14	0.829	48949
2	082620_MCL5_notx_01007-0002	5995	15.549	10	42	0.787	93215
3	082620_MCL5_notx_01007-0002	5316	20.894	12	53	0.868	111072
4	082620_MCL5_notx_01007-0002	4213	10.755	9	17	0.831	45309

Save to excel for
intensity code

H2AX analysis

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



	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 (px²)

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 “Complete secondary staining steps”
2. View H2AX images on 7th floor microscope
3. Analyze H2AX images with batch analysis

HW due M1D4

(group) - schematic

Bio render

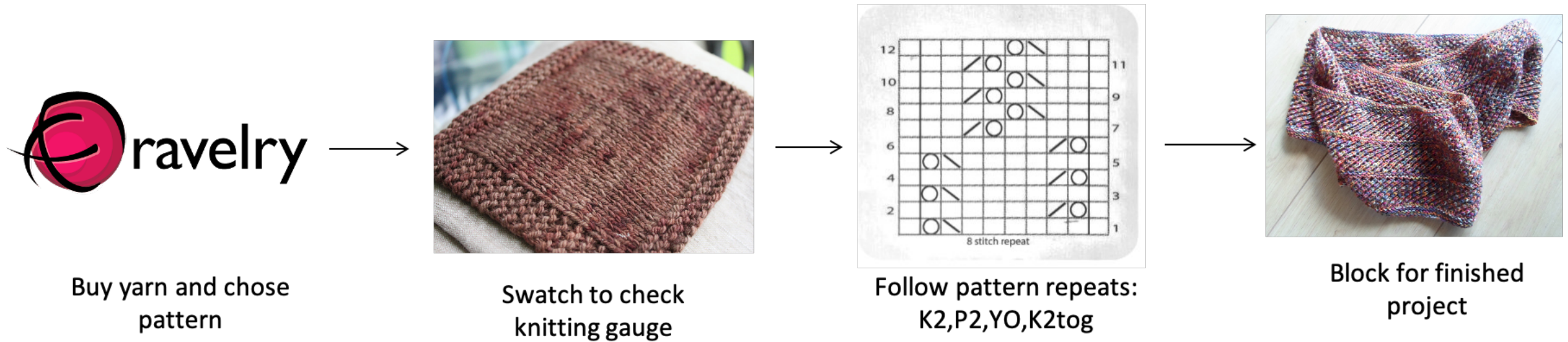
schematic
wiki
ppt slides

- Create an experimental schematic for the H2AX staining process (include analysis step)

(individual)

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

Schematic Example



workflow

⇒ **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

jargon

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

Results slide example

- 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

top 43-12 page
AC

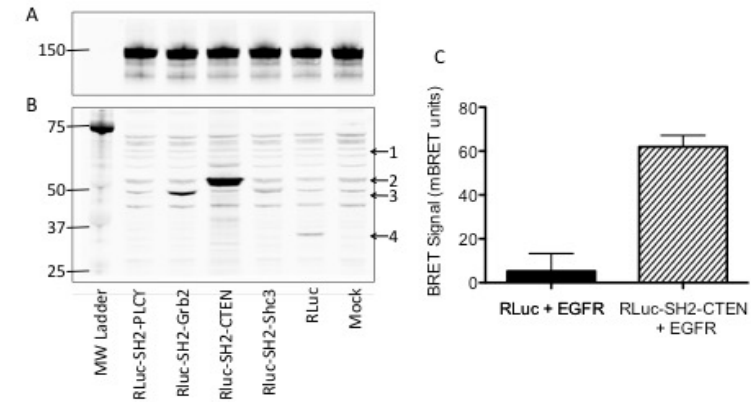


Figure 1: Development of BRET assay to monitor EGFR and SH2 domain interactions. CHO-K1 cells were transfected with Citrine-EGFR (A) and renilla luciferase (RLuc)-tagged SH2 domains from PLCg, Grb2, CTEN, and Shc3 (B). Western blots of CHO-K1 lysates were probed with anti-EGFR (A) or anti-RLuc (B) antibodies. 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.