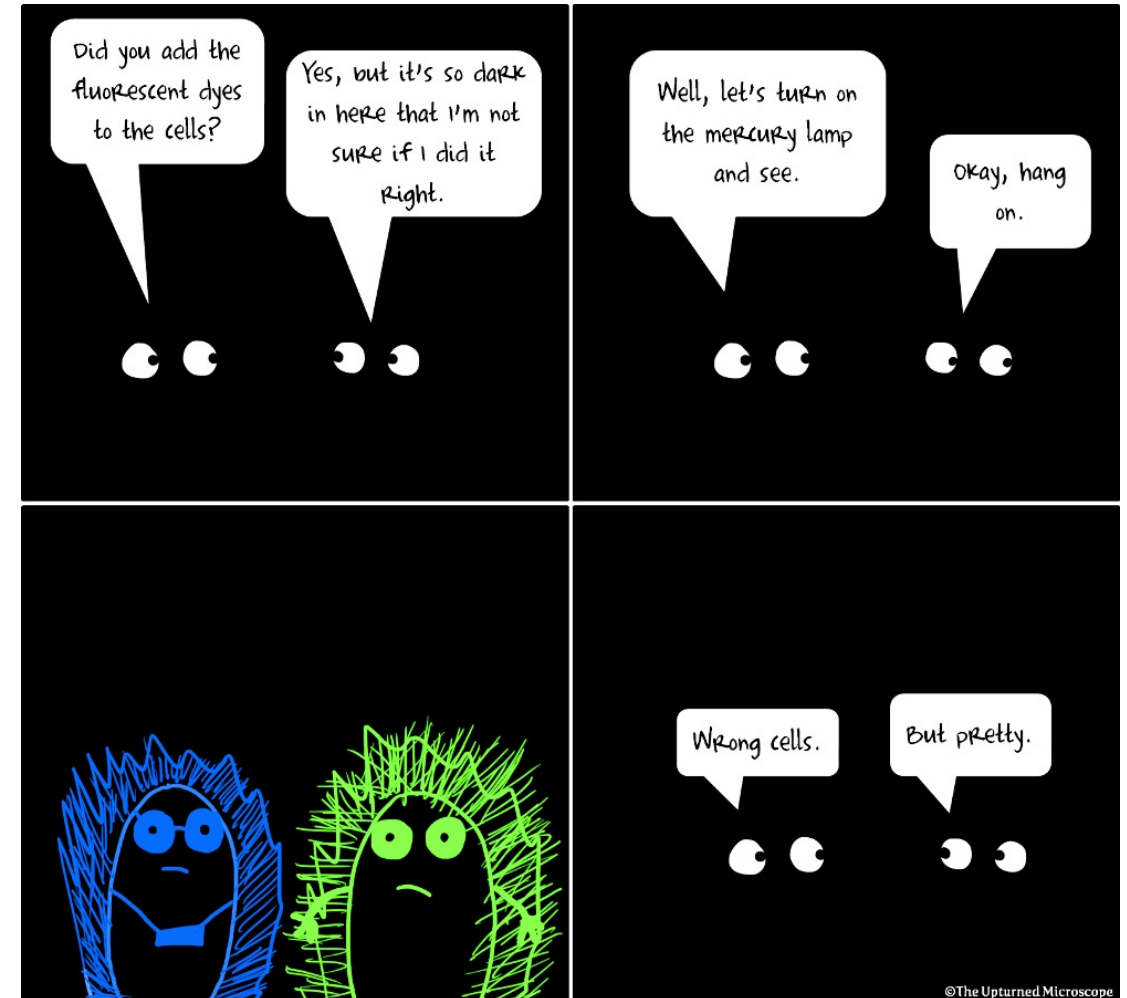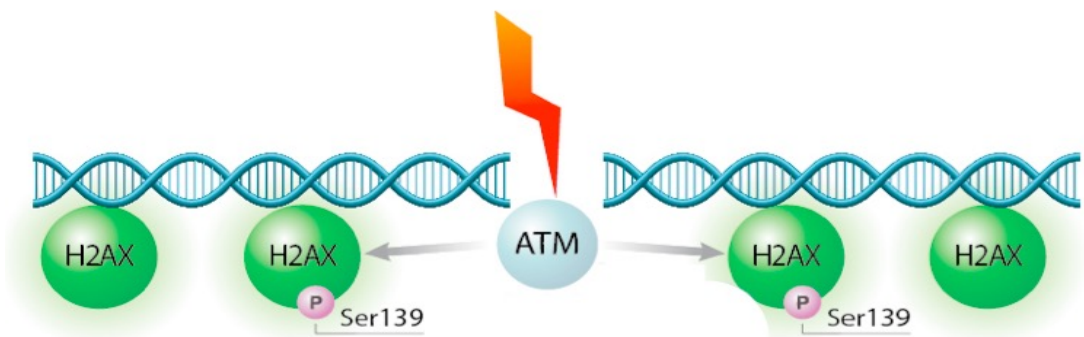# M1D3: Use immunofluorescence staining to assess repair foci experiment
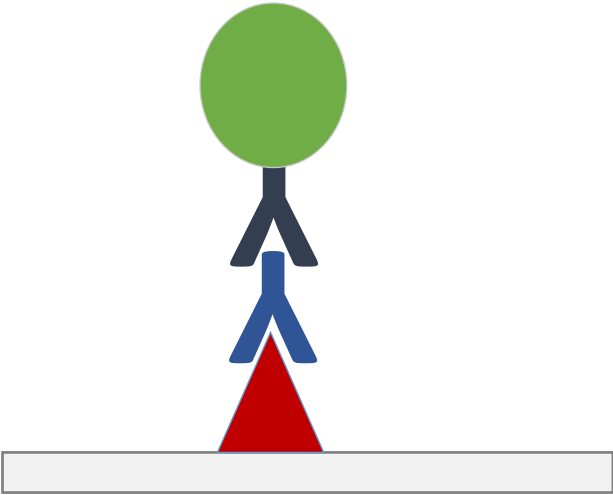
1. Comm Lab
2. Prelab
3. Antibody staining for γH2AX assay
4. Image analysis

Using immunofluorescence:
γH2AX assay to detect double-strand DNA breaks

- Histone H2AX phosphorylated at Ser139 if DSB
- Antibodies against γH2AX (phosphorylated form)

protein of interest	γH2AX
primary antibody	mouse anti-human anti-γH2AX
secondary antibody	goat anti-mouse
Fluorophore (conjugated to secondary antibody) 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
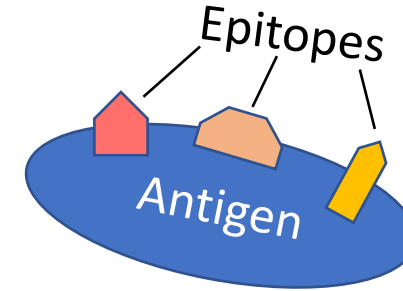
Primary antibodies vs secondary antibodies

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

- 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!

Epitopes

Antigen

epitope of an antigen

antigen

Fab

Fab

light chain

Fc

heavy chain

S-S bond

Gary E. Kaiser

Primary antibody

Secondary antibody conjugated to a fluorophore
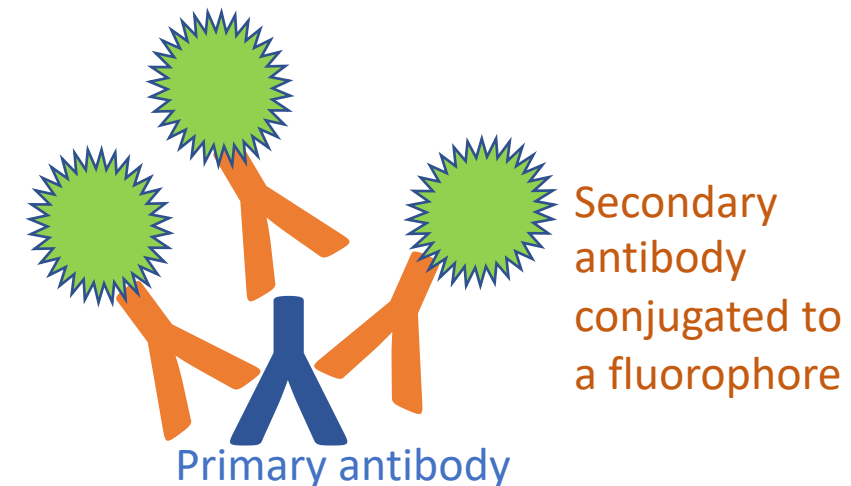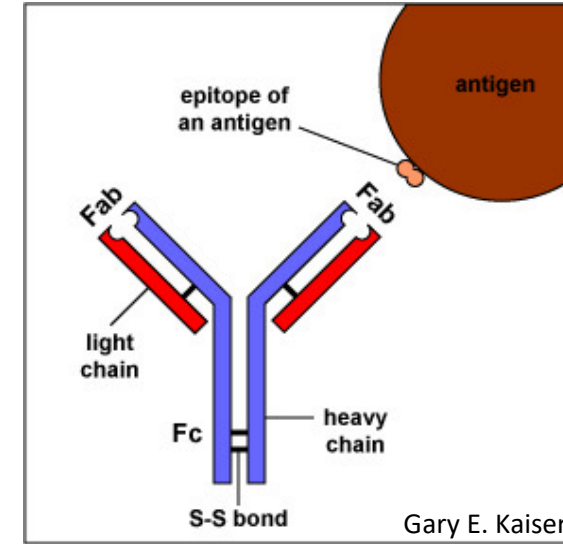
Using immunofluorescence (IF): steps in protocol
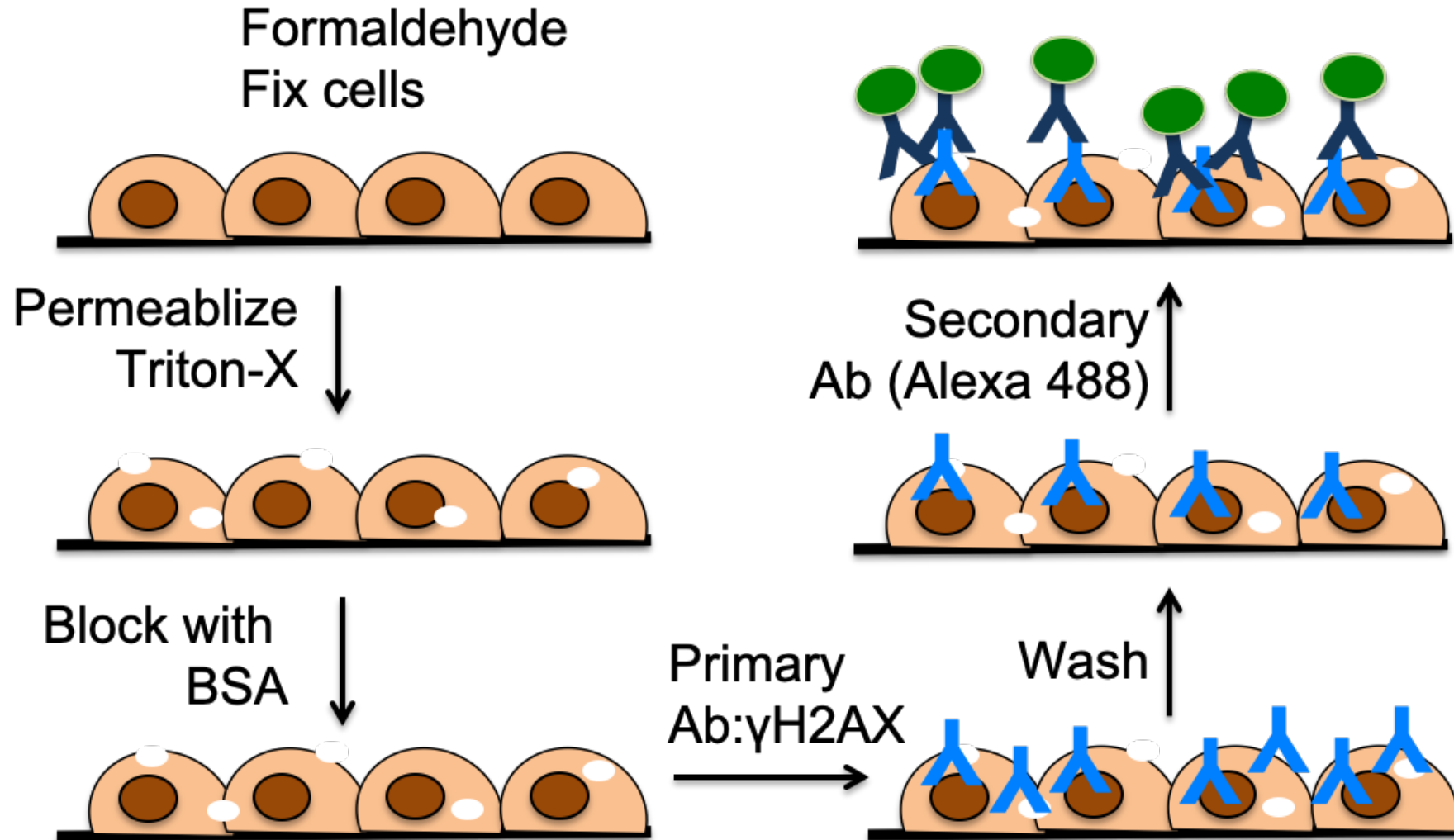
Finish IF by adding DAPI, then mount slides for imaging

Add DAPI
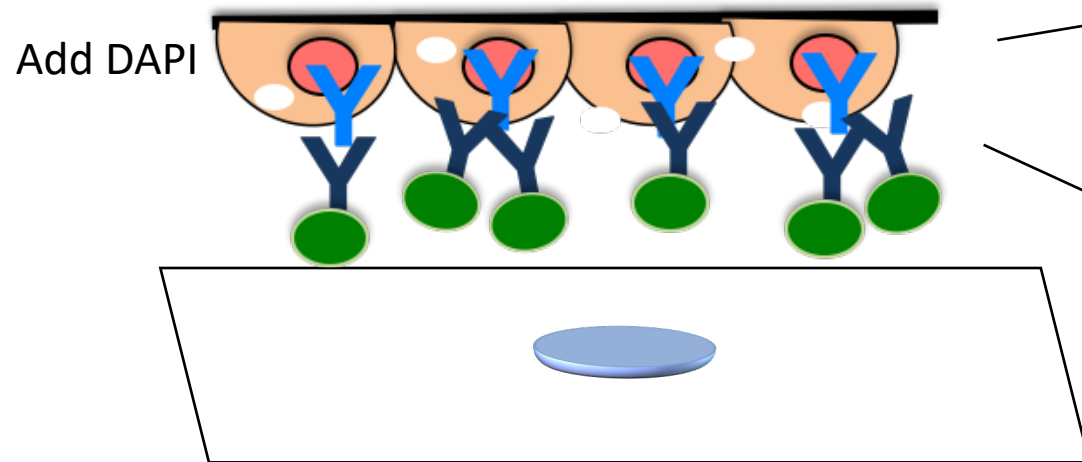
Mount coverslip on glass slide
with mounting media
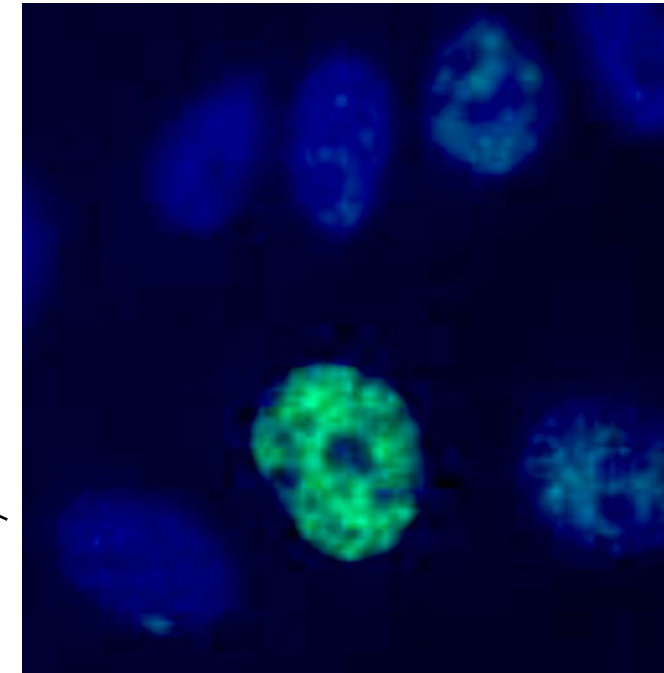
Blue= DAPI
Green= γH2AX

How will we analyze our images?

ImageJ

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

Two types of ImageJ analysis

1. Intensity
   - Measure average FITC signal intensity per nuclei

2. Foci counting
   - Measure average FITC punctae number per nuclei

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

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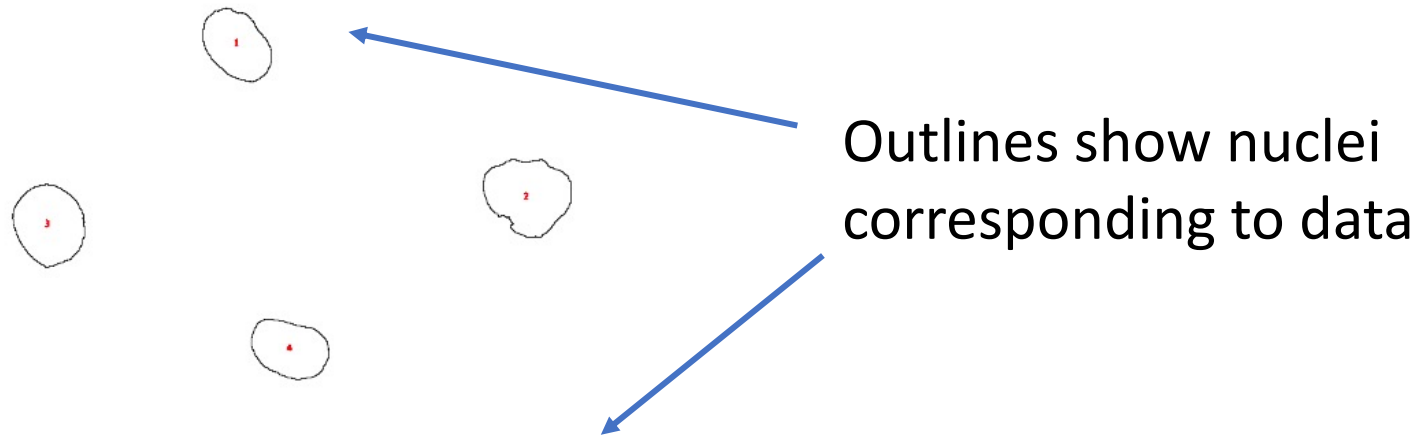
	Label	Area	Mean	Min	Max	Circ.	IntDen
1	082620_MCL5_notx_01007-0002	4291	11.407	10	14	0.829	48949
2	082620_MCL5_notx_01007-0002	5995	15.549	10	42	0.787	93215
3	082620_MCL5_notx_01007-0002	5316	20.894	12	53	0.868	111072
4	082620_MCL5_notx_01007-0002	4213	10.755	9	17	0.831	45309

Results

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 (px^2)

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 7th floor microscope
3. Analyze H2AX images with batch analysis

HW due M1D4

(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

- 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

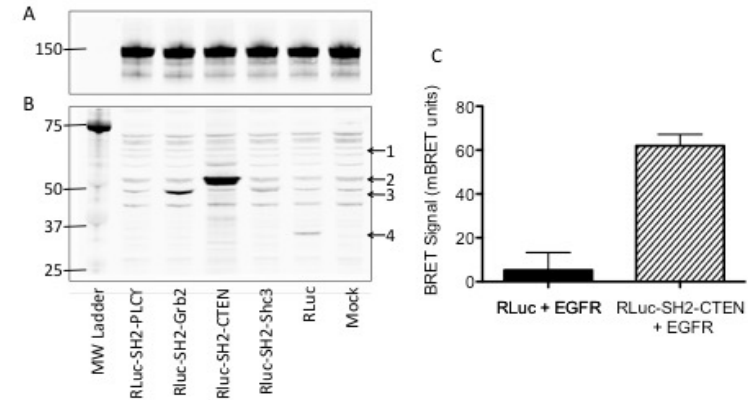- Bullet points **should** present and interpret the data

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
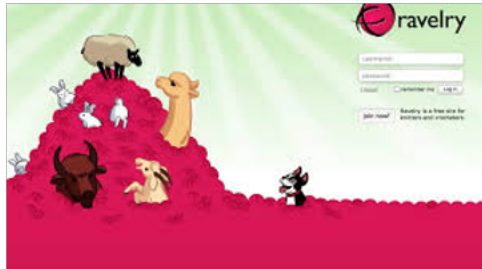
Choose a pattern

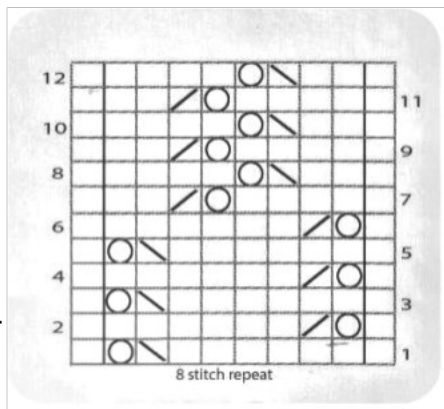Cast on 25 stitches

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:

Buy yarn and chose pattern

Swatch to check knitting gauge

Follow pattern repeats: K2,P2,YO,K2tog
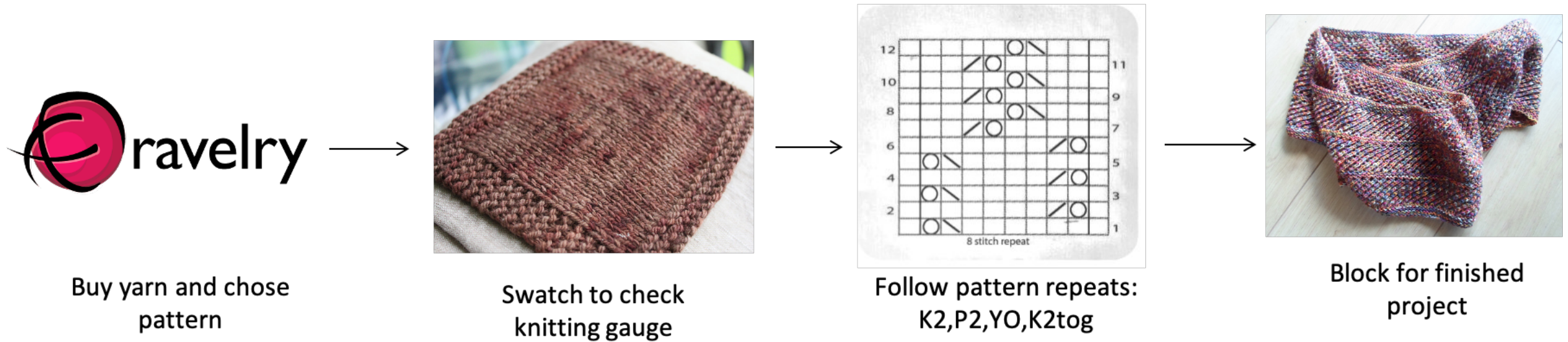
Block for finished project

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