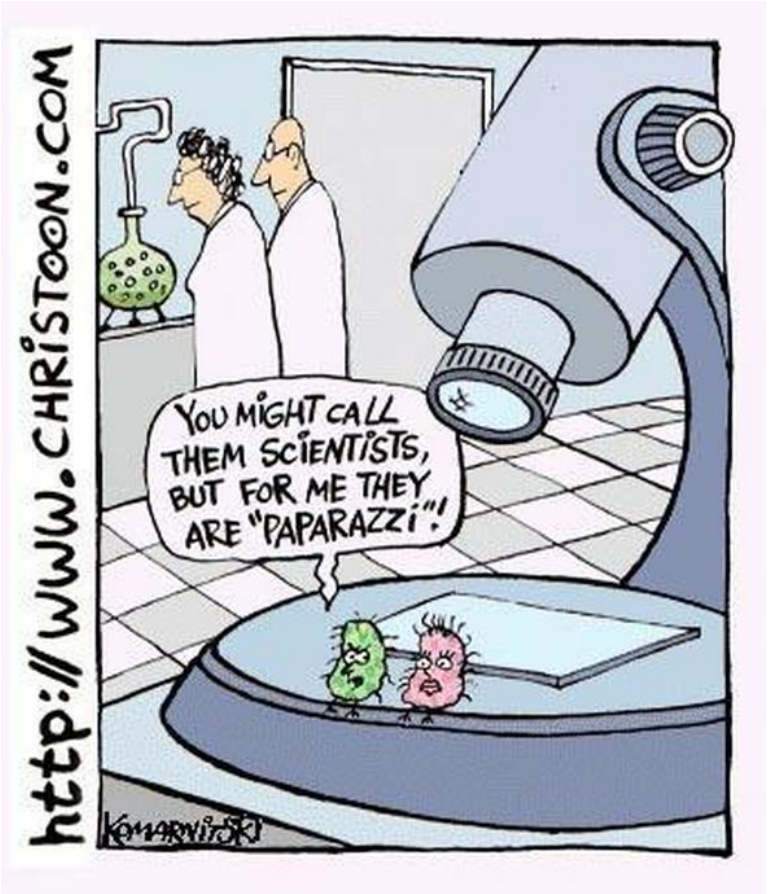
M1D4:
Image foci experiment and quantify results

1. Prelab discussion
2. Begin gamma-H2AX data analysis
3. Comm Lab workshop



Let’s review…

- Briefly, what are the key steps of the BER pathway?
- What is immunofluorescence (IF)?
- How did we fluorescently tag **γ**H2AX?

Considerations when using IF

Pros

- Multiple colors
- Low background & good contrast
- Molecular and spatial specificity
- Specialized techniques for 3D and high-resolution imaging

Cons

- Expensive equipment: laser, filters, sensitive cameras, …
- Toxicity to cells
- Need for fixing or gene manipulation

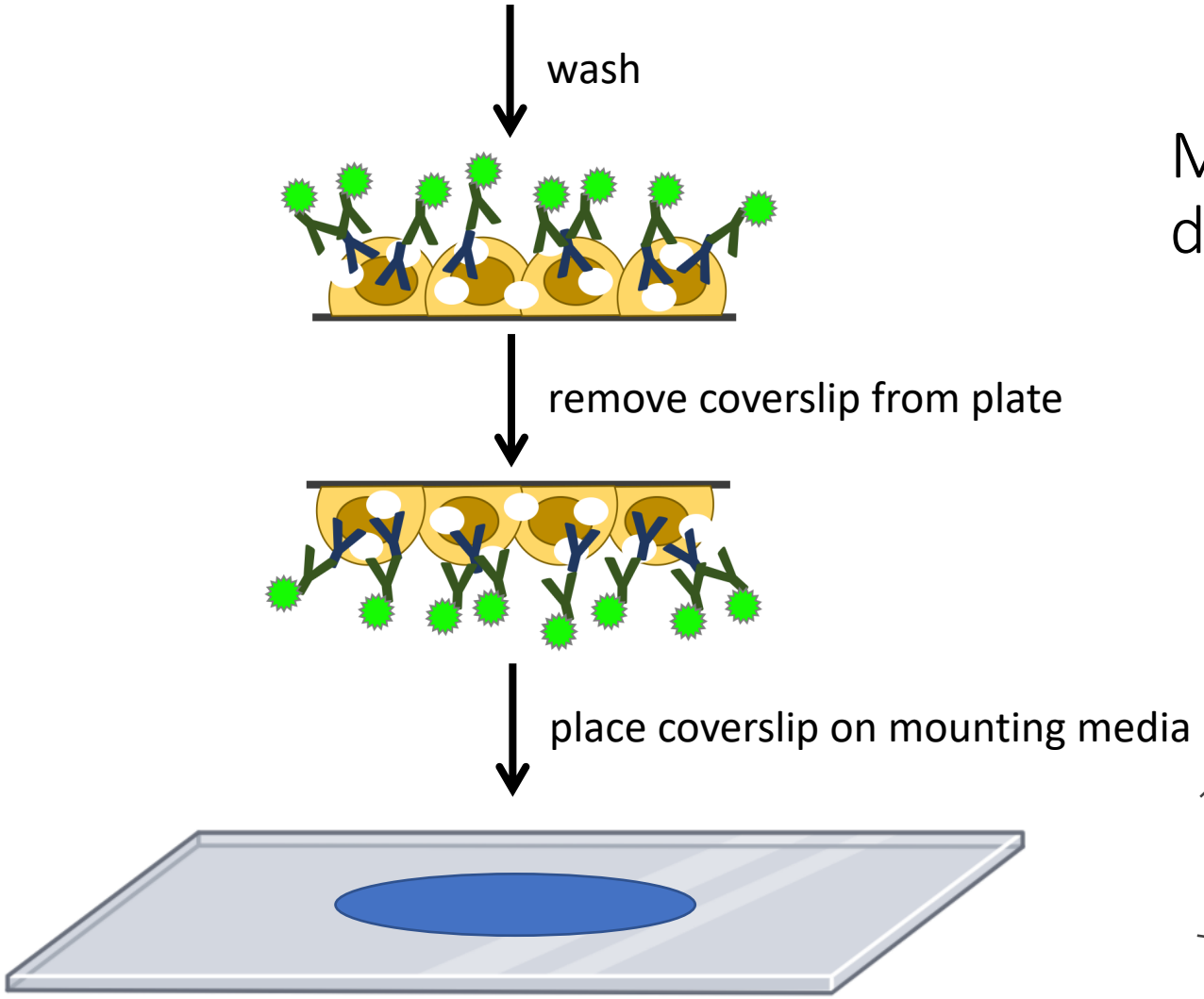
Nikon Small World Competition Winners

Left: (2003) Filamentous actin and microtubules (structural proteins) in mouse fibroblast (cells)

Middle: (2019) Fluorescent turtle embryo

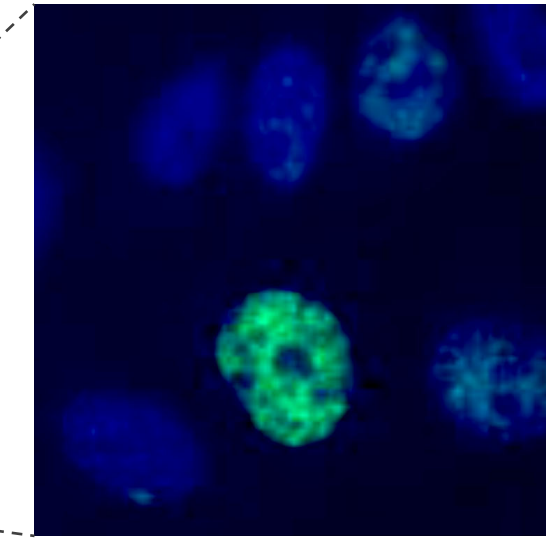
Right: (2017) Immortalized human skin (HaCaT keratinocytes) expressing fluorescently tagged keratin

How do we prepare for imaging γH2AX foci?



Mounting media contains 4′,6-diamidino-2-phenylindole (DAPI)

blue = DAPI
green = γH2AX



Demonstration of coverslip mounting procedure

Details for γH2AX imaging

- What controls are good to include in antibody staining experiments?

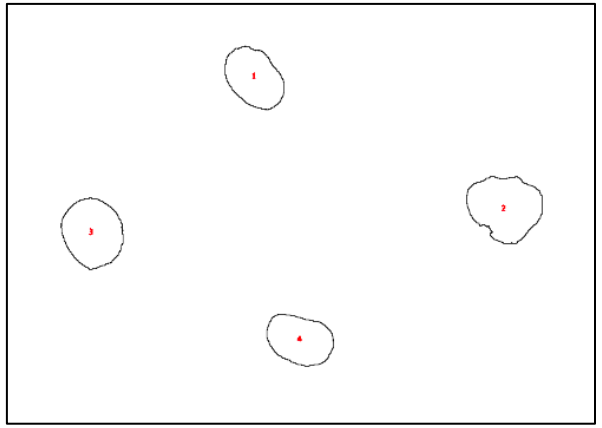
- What channels were used to image γH2AX experiment?

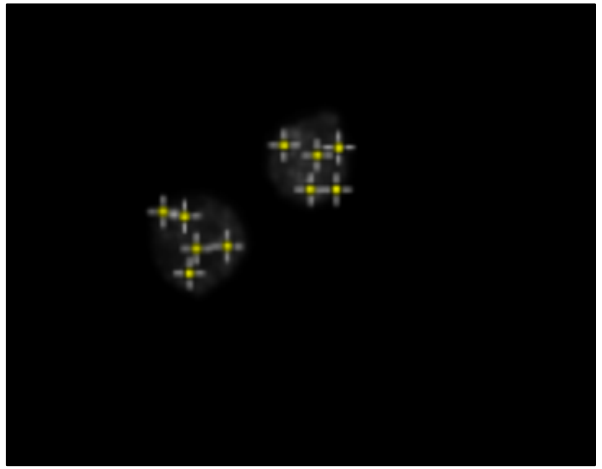
How will we measure DNA damage?

Two types of analysis will be completed:

1. Fluorescence intensity
   - Measure average FITC signal intensity per nuclei

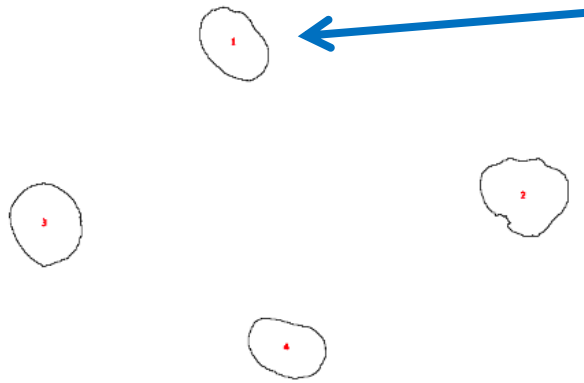
2. Foci abundance
   - Measure average FITC punctae per nuclei





ImageJ used to quantify image data

- For fluorescence intensity analysis:
  - Set threshold and mask to only examine FITC signal within DAPI-stained nuclei
  - Transfer results into Excel for analysis



outlines indicate nuclei that are included in the data

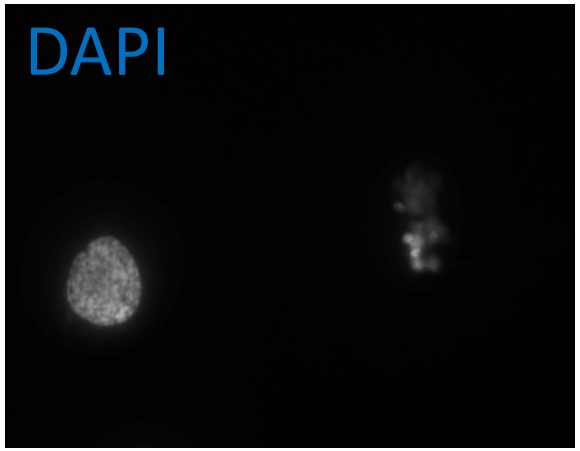
Results

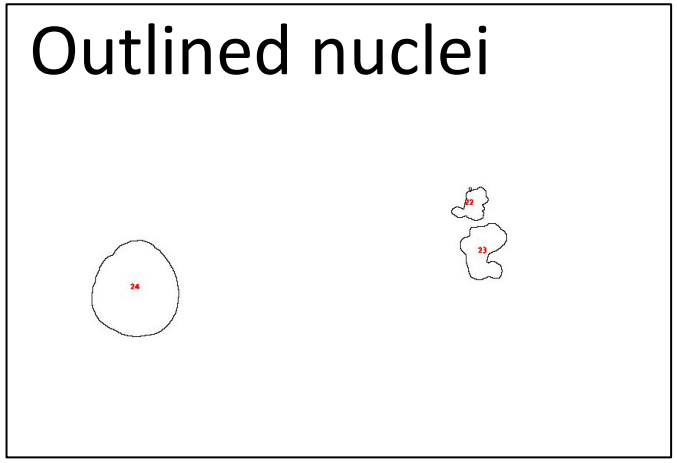
	Label	Area	Mean	Min	Max	Circ.	IntDen
1	082620_MCL5_notx_01007-0002	4291	11.407	10	14	0.829	48949
2	082620_MCL5_notx_01007-0002	5995	15.549	10	42	0.787	93215
3	082620_MCL5_notx_01007-0002	5316	20.894	12	53	0.868	111072
4	082620_MCL5_notx_01007-0002	4213	10.755	9	17	0.831	45309

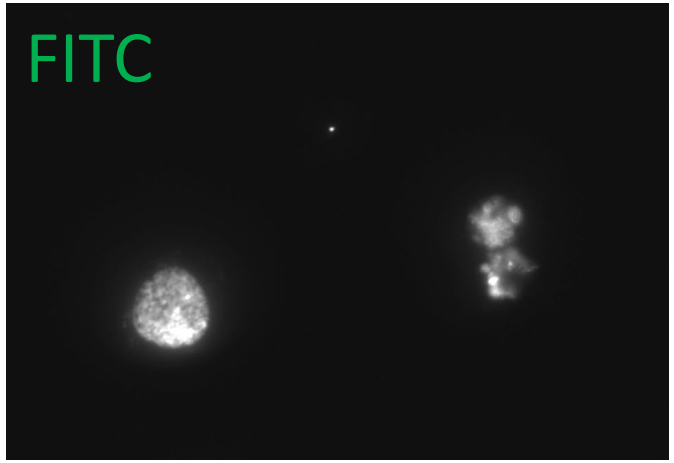
- For foci abundance analysis follow protocol from Duke University

Validate results by visually inspecting images

- Do the measured values make sense based on what you see?
- How much variability is observed between the cells?
- What are potential issues with the analysis methods?







Details for **γ**H2AX experimental setup

- What conditions were tested in your **γ**H2AX experiment?
  - Experimental condition(s):
  - Control condition(s):
- What types and how many replicates were included?
  - Technical:
  - Biological:

For today…

- Quiz due by 10 pm to Stellar!
  - Complete individually
- Work through gamma-H2AX data analysis
  - Record your notes in your laboratory notebook

For M1D5…

- Submit summary from BE Comm Lab appointment
- Draft figure of gamma-H2AX data and write associated text
  - Only required to use analysis from M1D4 Part 4 in this homework

Notes on figures for the Data summary…

Data Summary to be completed using **PowerPoint**

Each figure should relay one message

- Subpanels should be related to single conclusion
- Consider representative microscopy images and / or quantified representation of the data rather than a panel of multiple image examples
- Remember the title and caption

Text should be related to results in the figure

- See guidelines in homework description
- **Write in bullets!!**

Example for Results slide:

Image should not be the entire page

- Only needs to be large enough to be clear / visible

Title should be conclusive

- Don't state 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.
- Include details needed to "read" figure

Bullet points should present and interpret the data

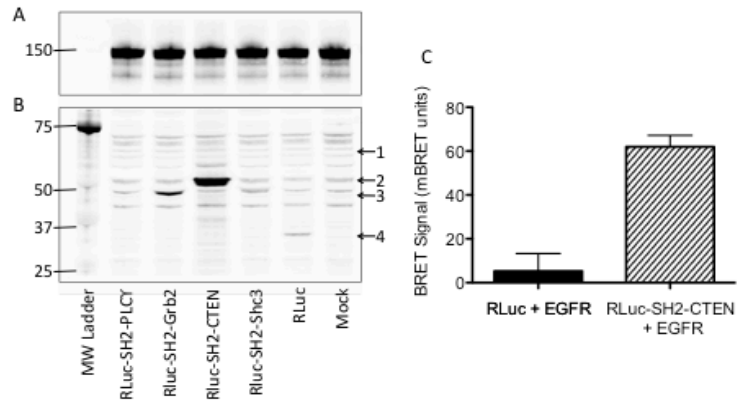


Figure 1: Development of BRET assay to monitor EGFR and SH2 domain interactions. CHO-K1 cells were transfected with Citrine-EGFR (A) and renilla luciferase (RLuc)-tagged SH2 domains from PLCg, Grb2, CTEN, and Shc3 (B). Western blots of CHO-K1 lysates were probed with anti-EGFR (A) or anti-RLuc (B) antibodies.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