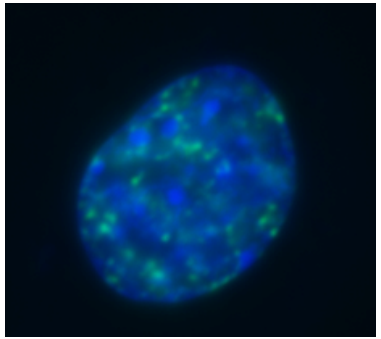
# M1D4: Complete Gamma-H2AX assay staining and begin Comet Chip with DNA damaging agents 09/24/19

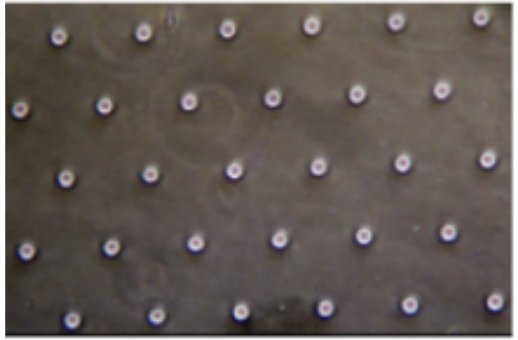
1. Quiz
2. Pre-lab part #1
3. Load Comet Chip and treat with MMS
4. Pre-lab part #2 during incubation
5. Complete Gamma-H2AX assay
6. Treat comet chip with Arsenite following MMS, lyse

# Overview of Module 1: Measuring Genomic Instability

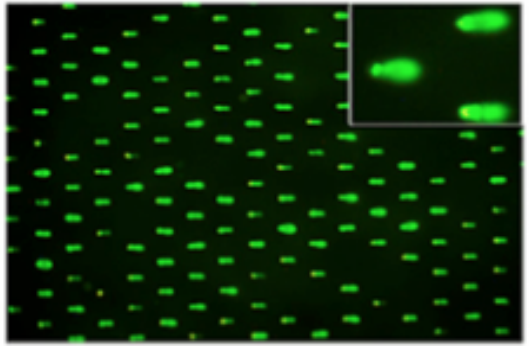
**Aim: Evaluate effect of Arsenic exposure on methylation induced base excision repair (BER)**



γH2AX assay

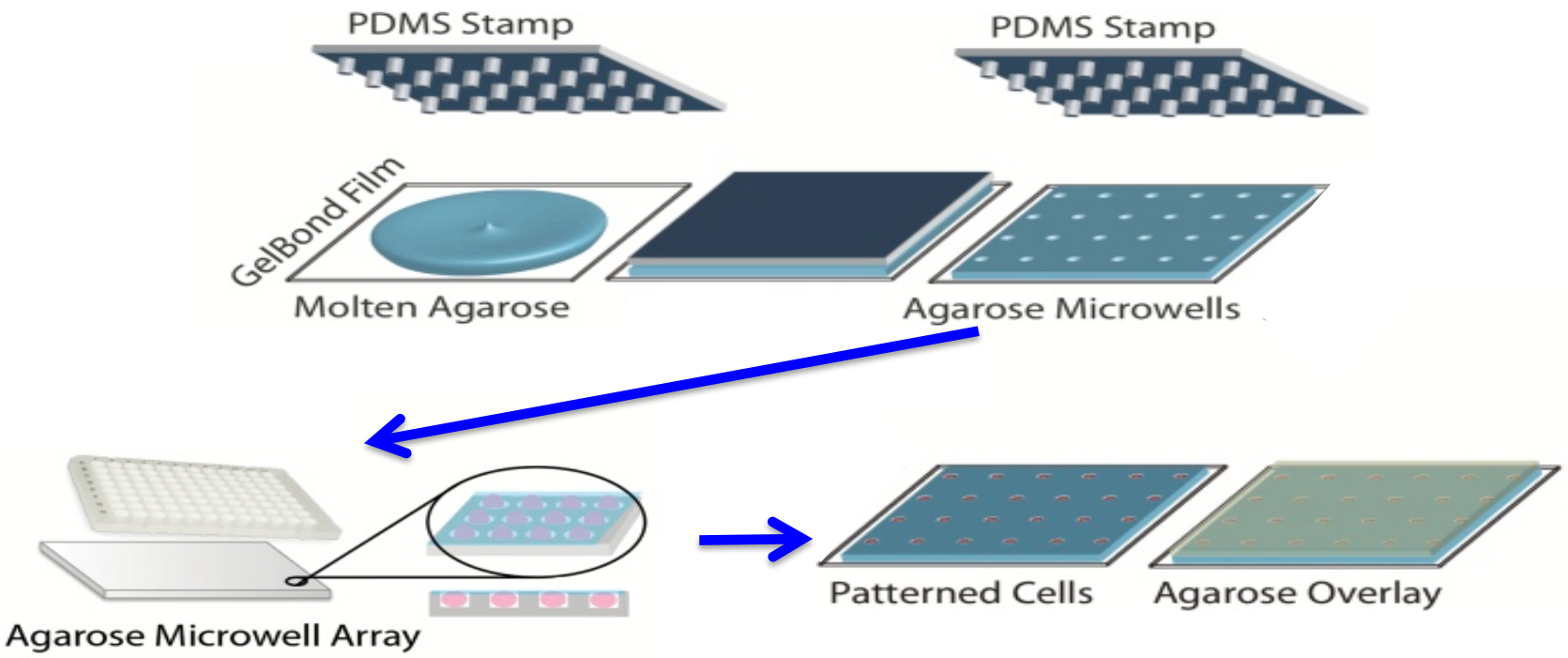


Optimize CometChip loading



CometChip assay

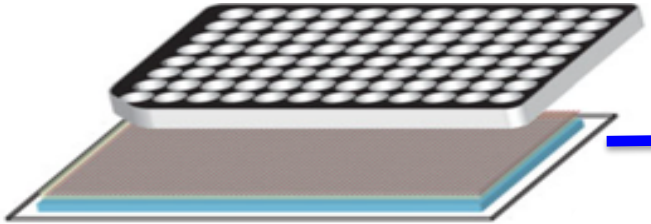
# Overview of CometChip Assay: Stamping microwells and loading cells




**What is the minimum # of CHO cells needed per macrowell?**

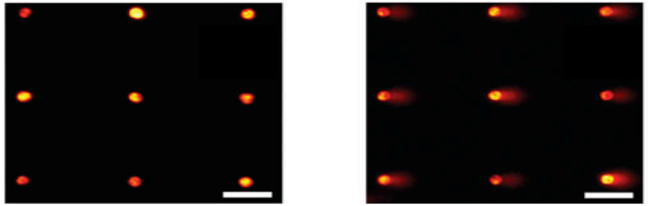
# Overview of CometChip Assay: Chemically treating cells and visualization

Treat captured cells in comet chip with MMS then As

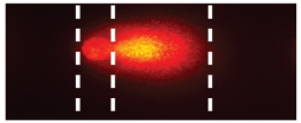


Lyse cells & unwind DNA (DNA still captured agarose in overlay)

Electrophoresis

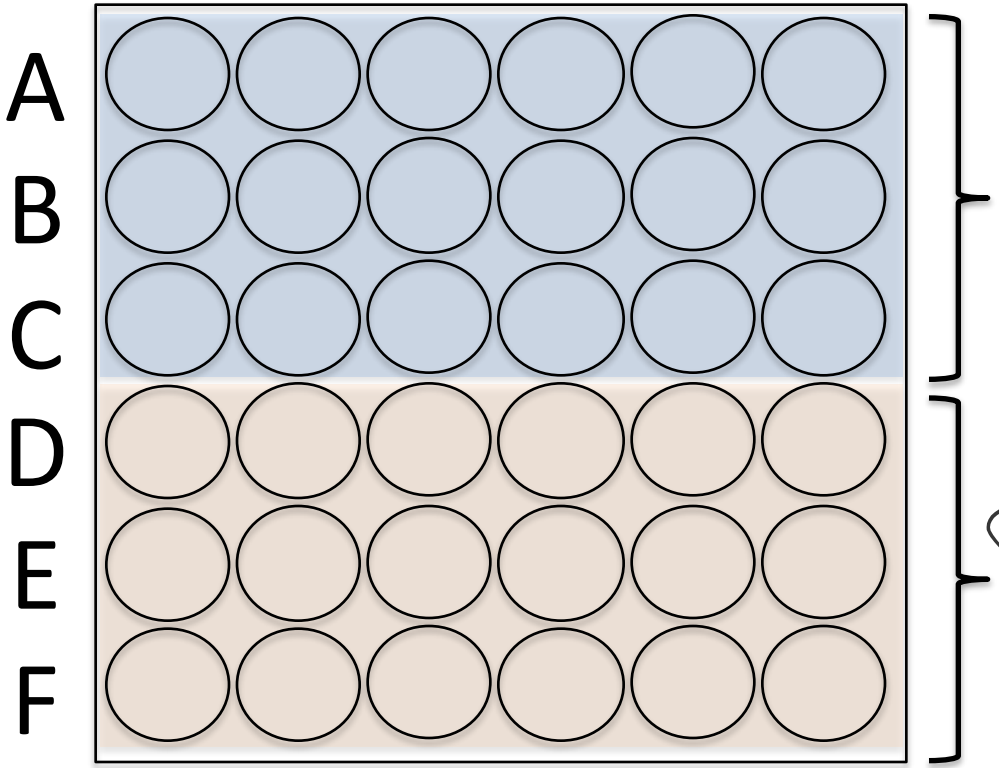
Stain DNA and image via fluorescence microscopy



Analysis via Matlab

# Keep track of the rows –

MMS treatment 1 hour, 37C in TC

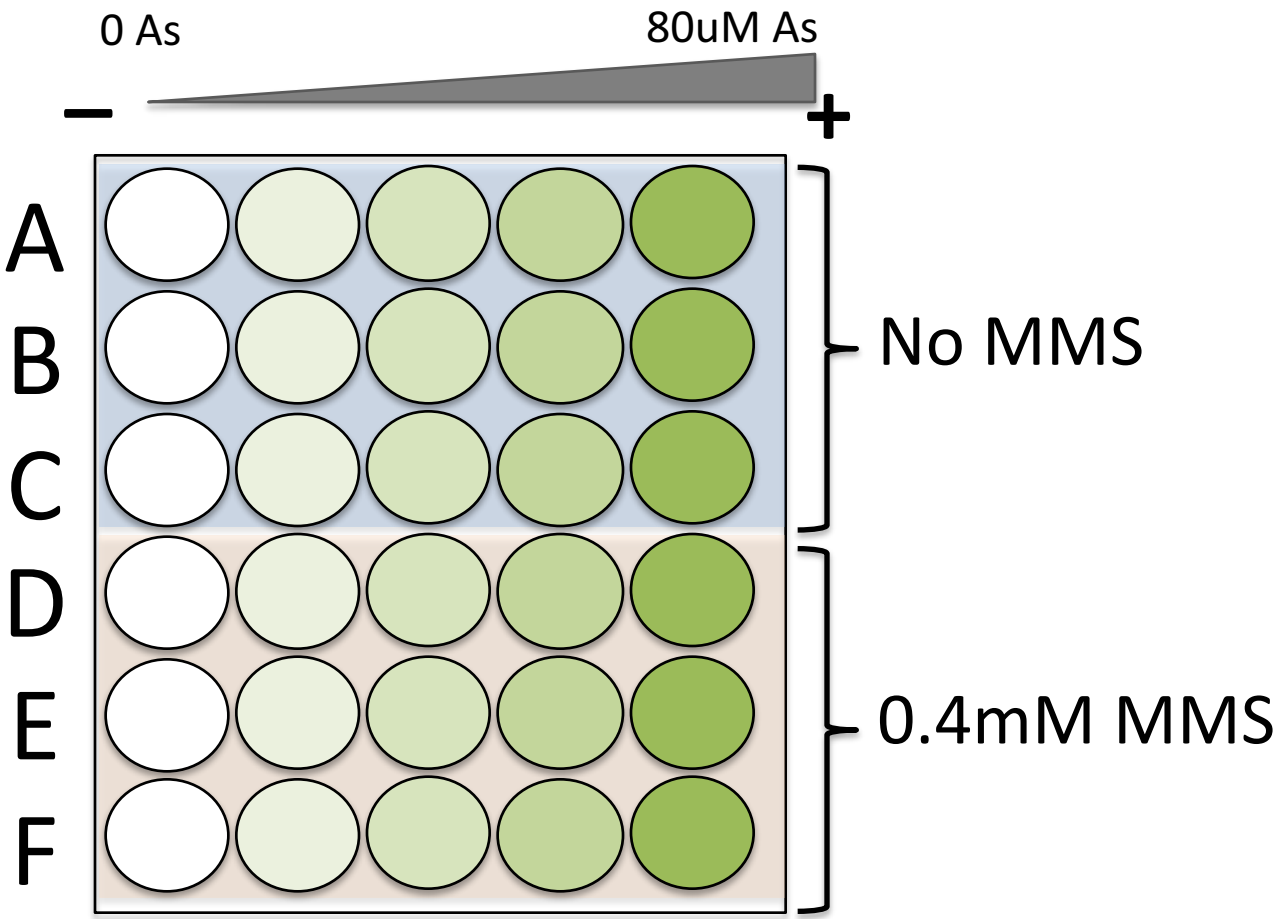



$C_1 V_1 = C_2 V_2$

Handling tips:

- MMS stock should be left on front bench, dilutions made in DMEM
- Minimize waste and collect all MMS!
- Must wear green flocked gloves, goggles

# Keep track of the columns – 5 concentrations!



# Preparing Arsenite dilution series:

Handling tips:

- Concentrated As should be left on front bench
- Dilutions made in DMEM
- Minimize waste and collect all!

Treat with: 0, 20, 40, 60, 80µM Arsenite

- 37°C for 2 hours
- Add **100µl** of drug dose to each macrowell
- **Triplicate**: each concentration will have three macrowells for each cell line
  - Make ___1___ mL of each concentration

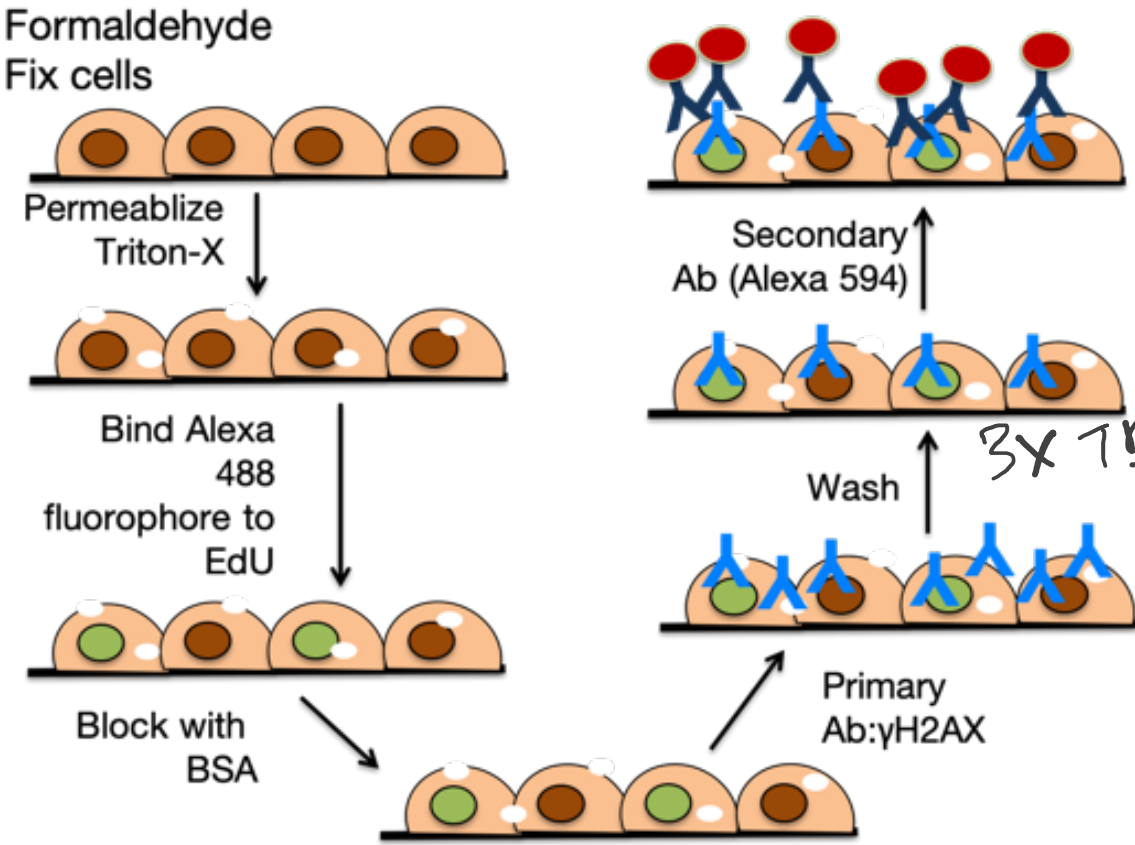
**Stock 1: 100 mM**

**1:1000, Stocks 2: 100 µM**

| | 0µM | 20µM | 40µM | 60µM | 80µM |
|---|---|---|---|---|---|
| Stock 2 | | | | | 800µl |
| DMEM | | | | | 200µl |

1mL

# Using immunofluorescence (IF) and EdU reaction:




3X TBS

Last week

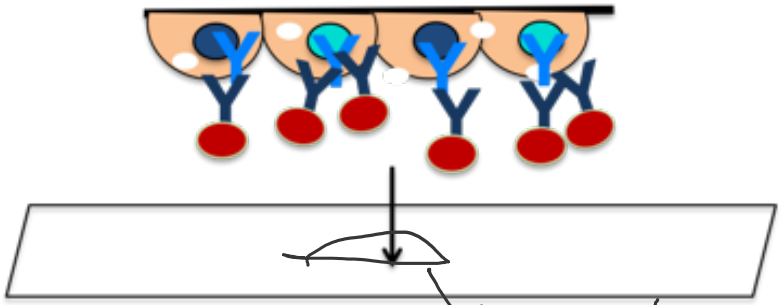
2° Ab Recognizes constant region of 1° Ab, Red fluor.

# Complete gamma H2AX staining, add DAPI stain and mount coverslips onto slide

Wash 3X TBS

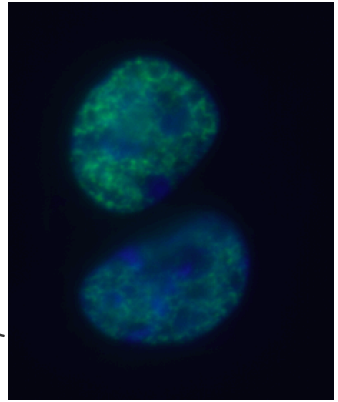
Add DAPI stain (all DNA stain)
to one wash after secondary antibody incubation

**Images from 60X objective**

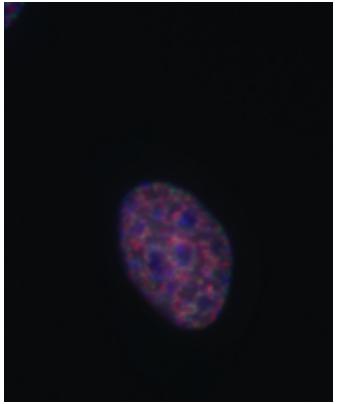


mounting media

Mount coverslip on glass slide



DAPI + EdU
(no γH2AX)



DAPI + EdU
+ γH2AX

can be done once w/ antibody

<u>Controls for antibody staining, what do we expect:</u>

Primary alone- check for background sig/interfere w/ other staining

Secondary alone- check for nonspecific binding

# Major assignments for Mod1

- Data summary draft
  - due by 10pm on Mon., October 14
  - revision due by 10pm on Sat., October 26

**Summary content**

1. Title
2. Abstract
3. Background & Motivation
4. Figures, Results & Interpretation
5. Implications & Future Work

# Example Results slide (from Wiki)

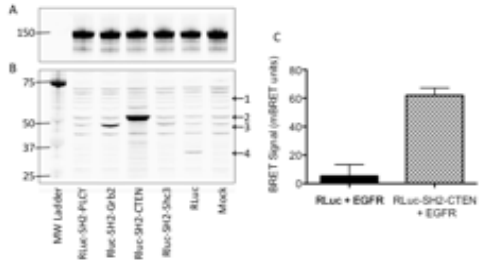



**Figure 1: Development of BRET assay to monitor EGFR and SH2 domain interactions.** CHO-K1 cells were transfected with Citrine-EGFR (A) and renilla luciferase (RLuc)-tagged SH2 domains from PLCg, Grb2, CTEN, and Shc3 (B). Western blots of CHO-K1 lysates were probed with anti-EGFR (A) or anti-RLuc (B)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

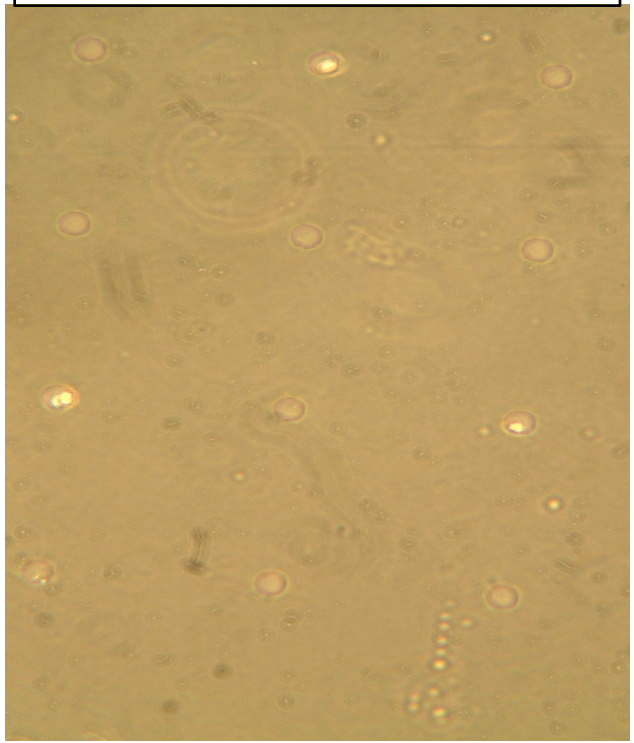
- To determine if the BRET system could be used to monitor EGFR activation, CHO-K1 cells were transfected with fluorescent EGFR and luciferase-tagged SH2 domains and a BRET assay was performed after growth factor stimulation.
- CHO-K1 were transfected with Citrine-EGFR in all conditions as indicated 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.

- PowerPoint format
- Limit figure size (1/3 of page)
- Caption describes image or graph
- Results text (2/3 of page) in bullet points

# Homework and analysis due M1D5

- Make a figure & caption
  - You should analyze and/or represent your light microscope images from M1D3
  - All figures **must include a title and a caption**.
  - Title: take away message from figure
  - Caption: info necessary to understand image/data in figure
- Receive homework credit for visiting Comm. Lab before M1D5!

**Images from 4X objective**



# In lab today

1. Load cells onto comet chip, start treatment with MMS
2. Complete staining of gamma-H2AX assay
3. Remove MMS and incubate comet chip with Arsenite 2 hours, followed by lysis

Name homework file:
LeslieM1D5hw_figure
LeslieM1D5hw_commlab

# HW due M1D5 (both individual)

1. Use the data from your cell loading experiment to create a figure, figure title and figure caption
   - Consider how you will represent the data and the size of the figure
2. Write a short summary of your communication lab appt. (1-2 paragraphs)