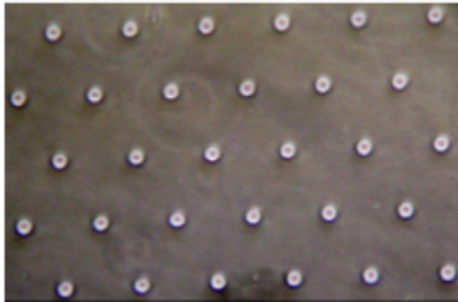
M1D3: Induce DNA damage with drug

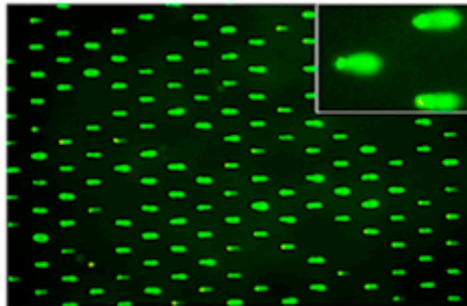
09/21/2016

Overview of “M1: Measuring Genomic Instability”



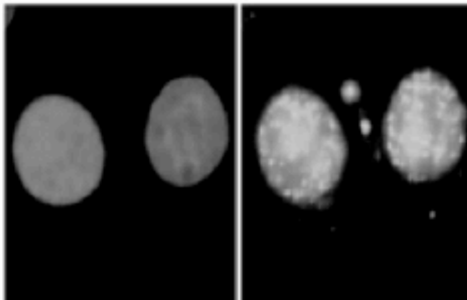
1. Optimize comet chip assay

- Test loading variables — discuss



2. Use comet chip assay to measure DNA damage / repair

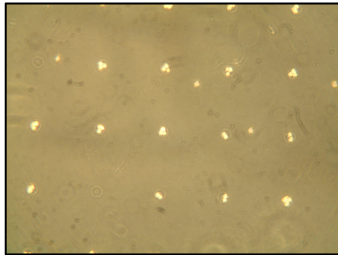
- Measure effects of MMS and H_2O_2 on BER — today
- Assess repair variability in healthy individuals — next week

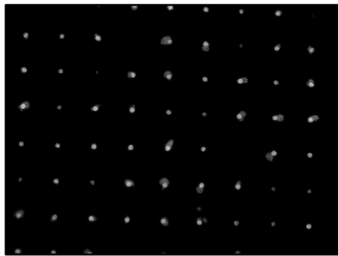


3. Use immuno-fluorescence assay to visualize DNA repair

- Examine effect of H_2O_2 on DSB abundance

Our afternoon





- Quiz
- CometChip loading discussion
- Pre-lab
- Load CometChip & induce DNA damage
- Doubling time discussion   or thesis defense?!
- CometChip electrophoresis

No lab Friday! Career fair !

Loading: how many cells for how long?

- What are your conclusions from M1D2 loading experiments 1 and 2?

Blue team: 10,000 or 23,000 cells/macrowell 25 min > 5 min	few cells
Red team: 60,000 and 90,000 15 min and 30 min	3-4 cells, no significant difference no significant difference
Purple team: 50,000 and 100,000 5 min and 10 min	80% of microwells had cells variable,

Engelward Lab : 25,000 - 100,000 cells / macrowell
5 to 30 min

# cells loaded	time (min)		
Yellow Team		fluo. int. (a.u.)	FI/1600
120,000	40	5242	3
120,000	40	5312	3
30,000	40	6381	4
30,000	20	4532	3
Green Team			
200,000	30	6257	4
200,000	30	6379	4
150,000	30	4980	3
200,000	15	4861	3
Blue Team			
100,000	35	4451	3
100,000	35	8444	5
200,000	35	10120	6
100,000	15	6951	4
Pink Team			
40,000	30	9603	6
75,000	30	8868	6
75,000	30	8820	6
75,000	5	4044	3
Purple Team			
150,000	20	4044	3
150,000	20	4061	3
15,000	20		
150,000	5		

Loading TK6: how many cells for how long?

- What can we learn from T/R students?

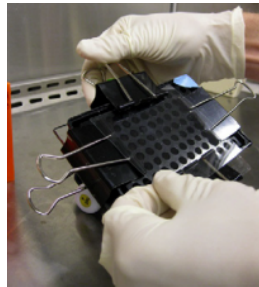
In lab today

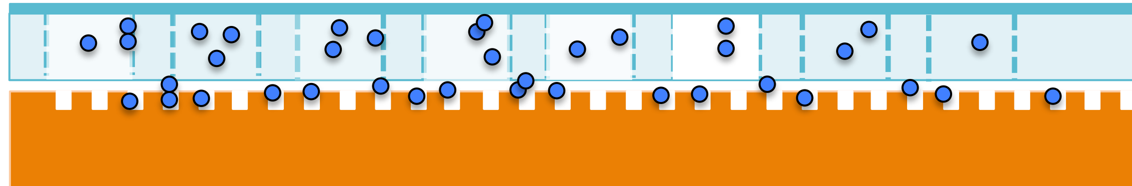
- Which loading condition was best?
- Load TK6:
  - 90,000 cells/well
  - for 15 min

from suspension at 500,000 cells/mL
15 mL of this suspension

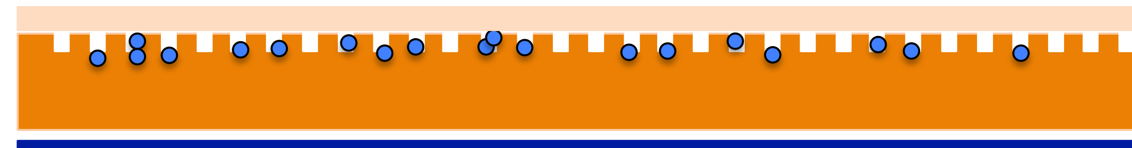
- Overlay with 1% LMP agarose
  - 3 min at room temperature + 3 min at 4 °C
- Treat 2 CometChips with MMS drug
  - one prepared by faculty, with “dead” (already lysed) cells
    - 50,000 cells / well, 30 min at 37 °C
  - one prepared by you today, with live TK6 cells

Start with CometChip from M1D1…

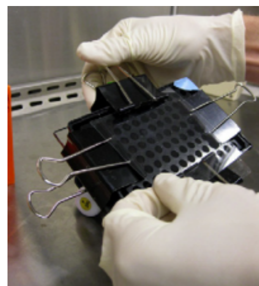


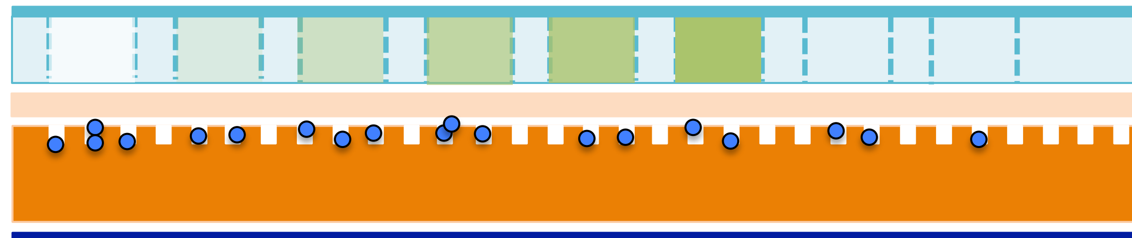


load TK6 cells



1% LMP agar

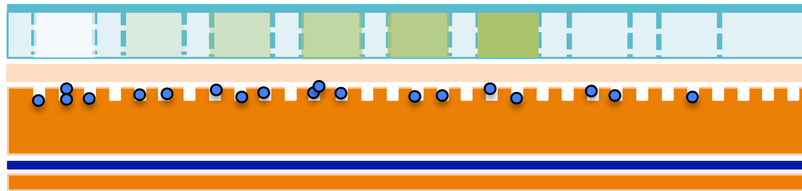




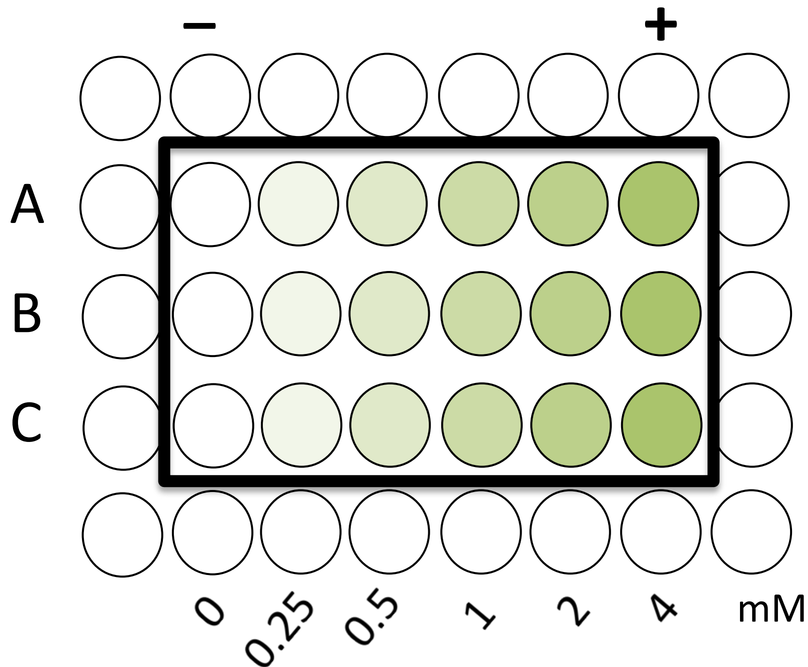
add drug

3 replicates

Treat cells with 6 different doses of MMS



drug treatment
(in macrowells of 96-well plate)



Methyl methanesulfonate is a mutagen!

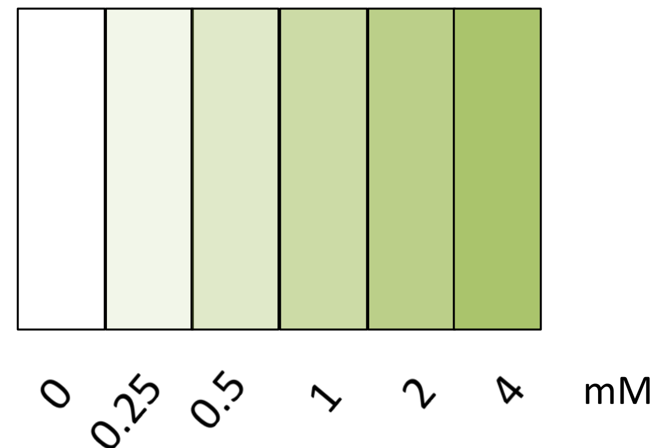
Wear flock-lined gloves (and lab coats) in addition to standard gloves

Special waste stream for *everything* in contact with MMS in fume hood

Treat cells with 6 different doses of MMS

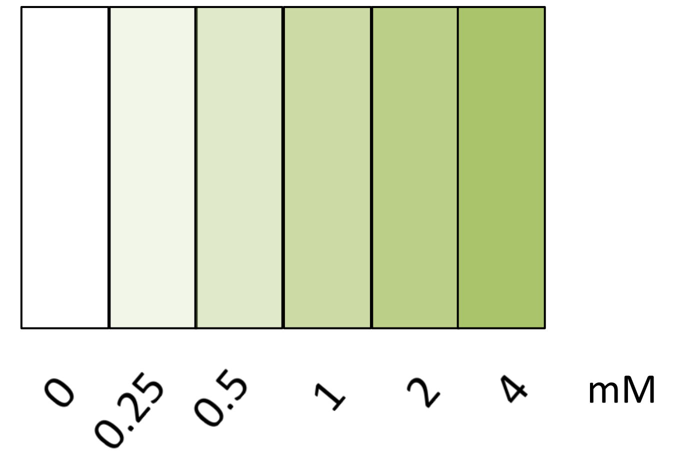
Preparatory dilutions

- MMS stock is at 80 mM
- Add 100 µL of drug dose to each macrowell
- 2x: Add drug to the “live” chip and “dead” chip in parallel
- Prepare dilutions in media
  - no FBS
- Treat with : 0, 0.25, 0.5, 1, 2, and 4 mM
- Dilutions should minimize waste!



6 different doses of MMS

Preparatory dilutions



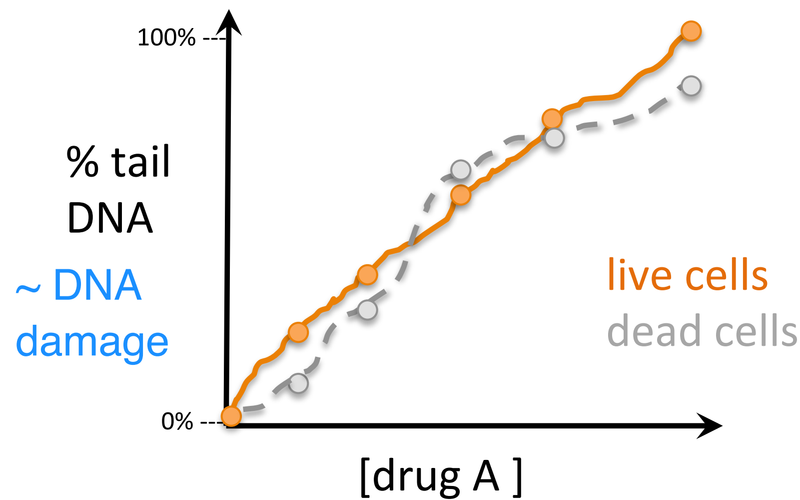
- MMS stock is at 80 mM
- Add 100 µL of drug dose to each macrowell,
  - so need 600 µL of each dose
- Prepare: 0, 0.25, 0.5, 1, 2, and 4 mM

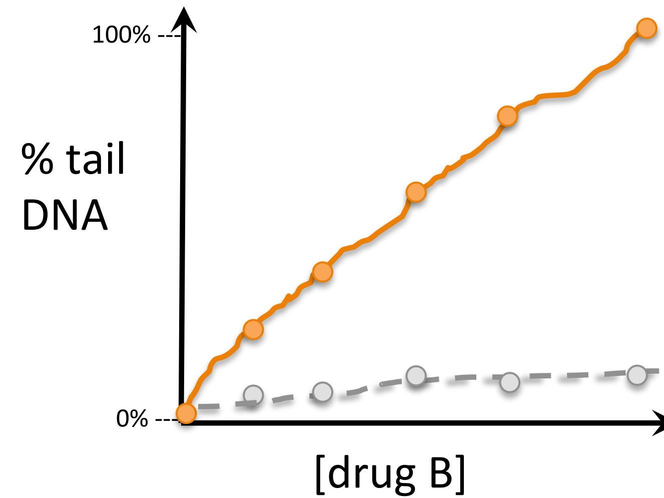
1. Make 8 mM stock: 2.25 mL medium + 250 µL of {80 mM stock}
2. Dispense 1.25 mL medium to all reservoirs
3. Serial dilution:
   - Reservoir 4: add 1.25 mL of {8 mM}
   - Reservoir 2: add 1.25 mL of {4 mM}
   - Reservoir 1: add 1.25 mL of {2 mM}
   - Reservoir 0.5: add 1.25 mL of {1 mM}
   - Reservoir 0.25: add 1.25 mL of {0.5 mM}
   - Reservoir 0: add 1.25 mL of medium
4. Incubate 30 min at 37 °C

Why analyze dead cells?

Note: T/R students treated their TK6 cells with another drug, H_2O_2.

➢ Will the two chemicals have the same DNA damaging effect?

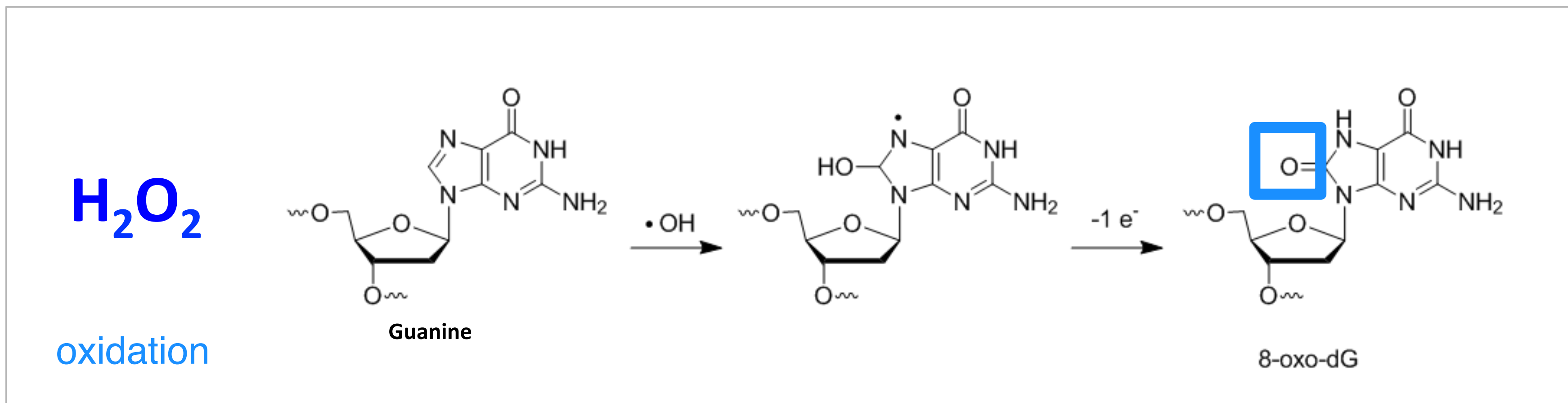


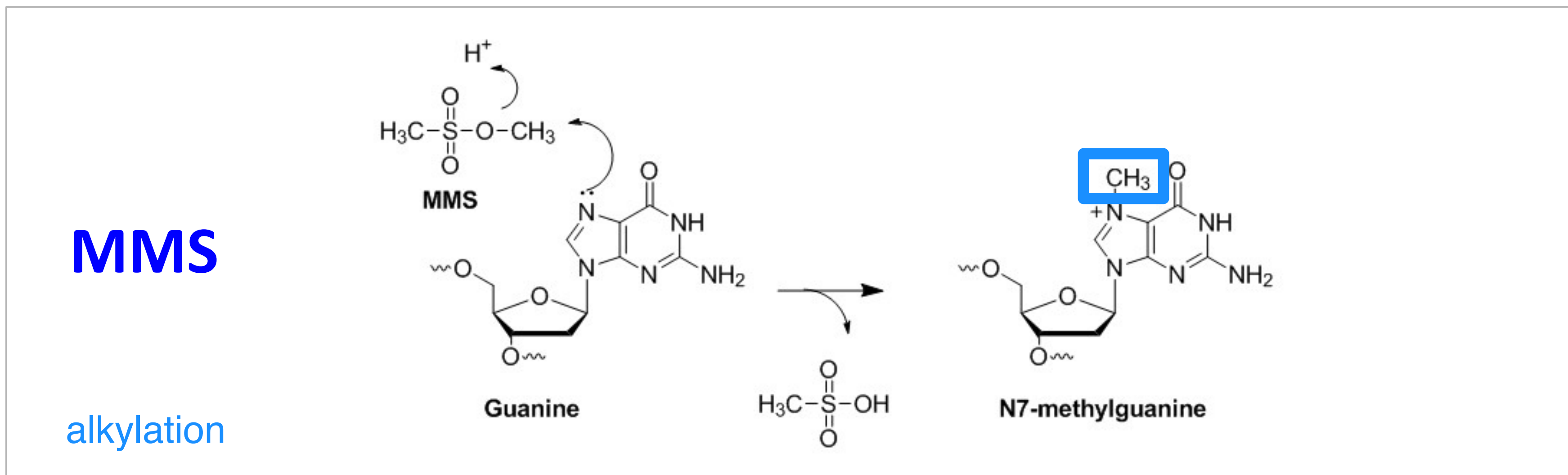


chemically vs. enzymatically

induced DNA damage

How do H_2O_2 and MMS damage DNA?





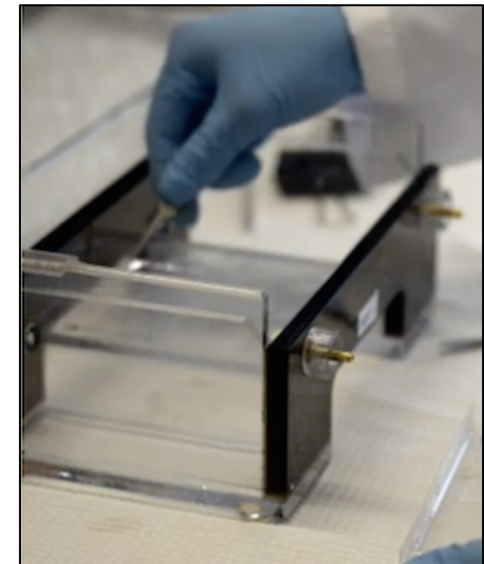
… to be continued next week!

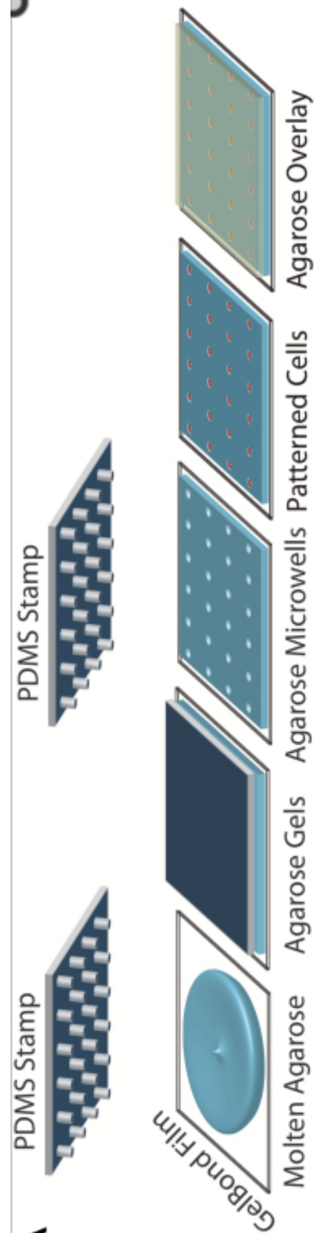
Lysis & electrophoresis of CometChips

EDTA: chelator that sequesters cations (enzyme cofactors among them)

- Alkaline lysis solution (> 1 h at 4°C)
  - 2.5 M NaCl, 100 mM Na_2EDTA, 10 mM Tris, (water)
  - pH 10 — unwind DNA
  - Triton X-100 — detergent/surfactant, disrupts cell membranes
- Electrophoresis buffer (40 min incubation + 30 min run, at 4°C)
  - 0.3 M NaOH, 1 mM Na_2EDTA
  - pH 13.5
  - double-sided tape
- Electrophoresis
  - 30 min
  - 1 V/cm (or 16 V for us) — very slow
  - 300 A
- Neutralize & dye (SYBR Gold)

because SYRB Gold is dim, quenched if pH<5 or >9





Tips to write Methods (due M1D4)

- Include enough information to replicate the experiment
  - List manufacturers information
  - Be concise and clear in your description
- Use subsections with descriptive titles
  - Put in logical order
  - Begin with topic sentence to introduce purpose
- Use clear and concise full sentences
  - Eliminate tables and lists
  - Passive voice expected
- Use the most flexible units
  - Write concentrations (when known) rather than volumes
- Eliminate '109 specific details
  - Do not include details about tubes and water!
  - Assume reader has some microbiology experience

Improving a Methods paragraph

<u>Tissue Culture:</u>

TK6 cells were grown in a T75 flask with 12 mL RPMI supplemented with 10% FBS. The cells were kept in an incubator at 37°C. A Trypan blue ~~stain was used to assess if the cells were alive or dead.~~

Improving a Methods paragraph

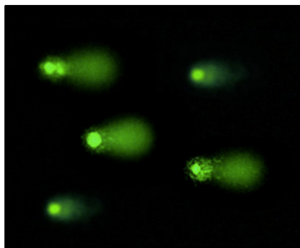
<u>Maintaining lymphoblastoid cell line(s):</u>

- TK6 human lymphoblastoids (gift of the Engelward Lab, MIT, Cambridge MA) and Coriell cells (GM15221, GM15242, and GM15061, Coriell Institute for Medical Research, Camden NJ) were cultured at 1-9 x 10^5 cells/mL in suspension in 1X RPMI medium 1640 (Invitrogen, Carlsbad CA) supplemented with 10% fetal bovine serum FBS (Invitrogen). All cell culture media were supplemented with 100 units/mL penicillin-streptomycin (Invitrogen).

Let's get started!

- Load TK6
- Overlay with 1% LMP agarose
  – 3 min at room temperature + 3 min at 4 °C

- Treat 2 CometChips with MMS drug
  – (enzymatically) dead
  – live



- Lyse 2 CometChips
- Electrophorese 2 CometChips

Thesis defense

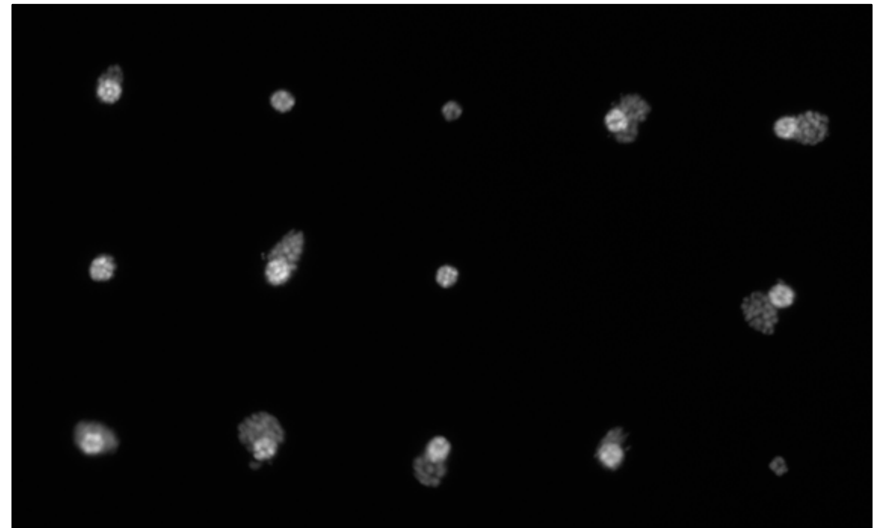
Wednesday, September 21st (today!)
3:30 PM
56-614

Investigation into the Role of DNA Damage and Repair during Influenza Infection and Inflammation

by Marcus C. Parrish
Engelward lab

Doubling time of TK6 cells – sample & images

- T = 0
- fixed overnight at 4 °C with 10% formalin (on Friday 09/16 at 5pm)
- labeled overnight at 4 °C with Vybrant Dye Cycle Green (Life Tech.)
- (not lysed)



- T = 2.5 days = 62 h
- fixed 2 h at 4 °C with 10% formalin (on Monday 09/19 at 7am)
- labeled overnight at 4 °C with Vybrant Dye Cycle Green (Life Tech.)
- (not lysed)

Doubling time of TK6 cells – Matlab analysis

	A1		A2		B1		B2	
day	2.5	0	2.5	0	2.5	0	2.5	0
average signal (a.u.)	82052	4451	82052	8444	85916	10120	70377	6951
doubling time (h)	15		20		21		19	
total DNA in microwell	123495	6479	123495	1065	150278	3949	85501	9122
(a.u. = fluorescence intensity)	92015	837	92015	2843	61555	3343	85621	7244
	96638	912	96638	3802	60232	9140	43434	3925
	141691	4347	141691	1652	61232	3821	89521	8736
	141351	3949	141351	2676	48203	9981	47265	7176
	168353	3836	168353	8527	25169	2311	34547	5509
	74210	3161	74210	1834	43736	10551	32500	5140

- Fluorescence signal intensity: 1600 a.u. ~ 1 cell

$$DoublingTime = \frac{ElapsedTime}{NumberOfDoublings} = \frac{T_{end} - T_{beginning}}{\log 2\left(\frac{\#cells_{end}}{\#cells_{beginning}}\right)} = \frac{62}{\log 2\left(\frac{signal_{t=2.5d}}{signal_{t=0}}\right)}$$

- Published TK6 doubling time = 20 h