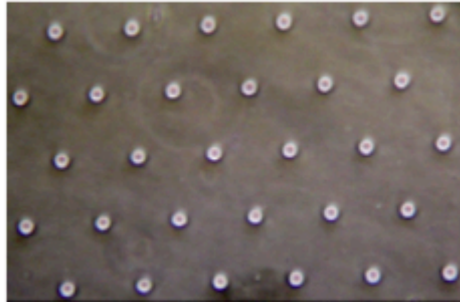
M1D3: Measure DNA damage with CometChip

09/20/16

1. Quiz
2. Pre-lab Discussion
3. CometChip loading discussion
4. Load CometChip, Induce DNA damage
5. CometChip electrophoresis

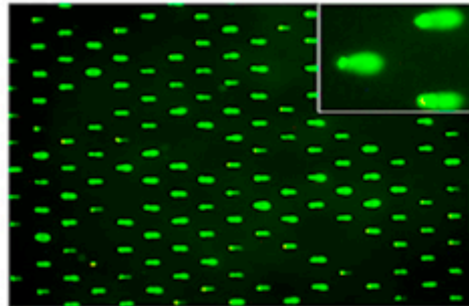
Overview of "M1: Measuring Genomic Instability"



1. Optimize comet chip assay

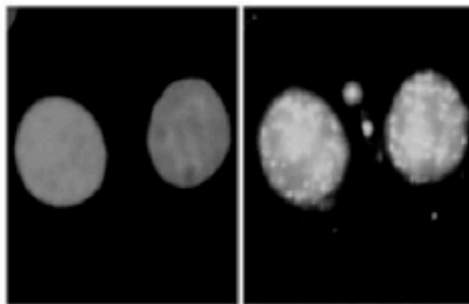
- Test loading variables

discuss results today



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

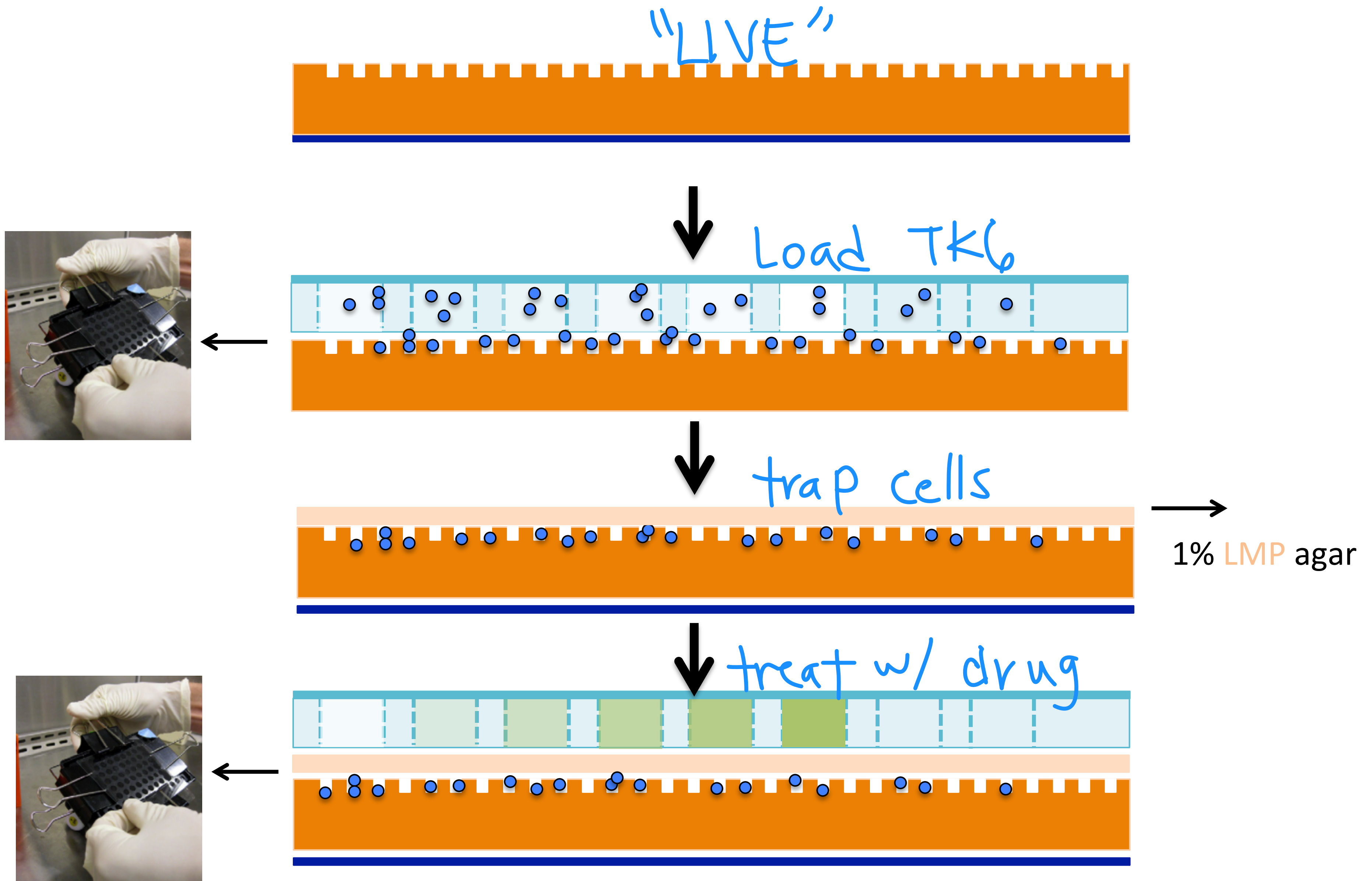
- Measure effects of MMS and H_2O_2 on BER
- Assess repair variability in healthy individuals → Next week



3. Use immuno-fluorescence assay to visualize DNA repair

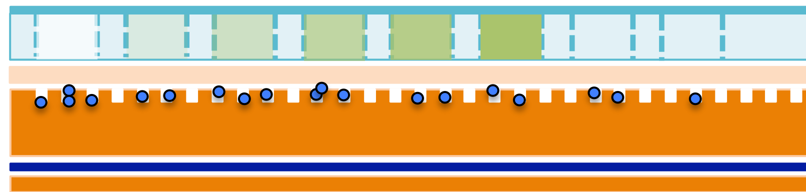
- Examine effect of H_2O_2 on DSB abundance

Start with CometChip from M1D1…

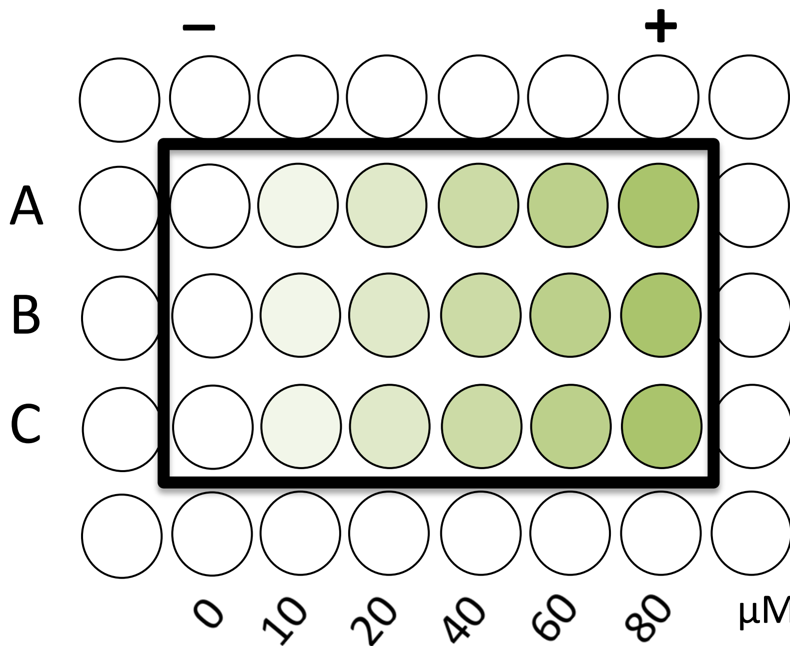


Treat cells with 6 different doses of H_2O_2

Concentrated H_2O_2 is a mutagen!
Wear gloves and a lab coat at all times.



H_2O_2 treatment
(in macrowells of 96-well plate)



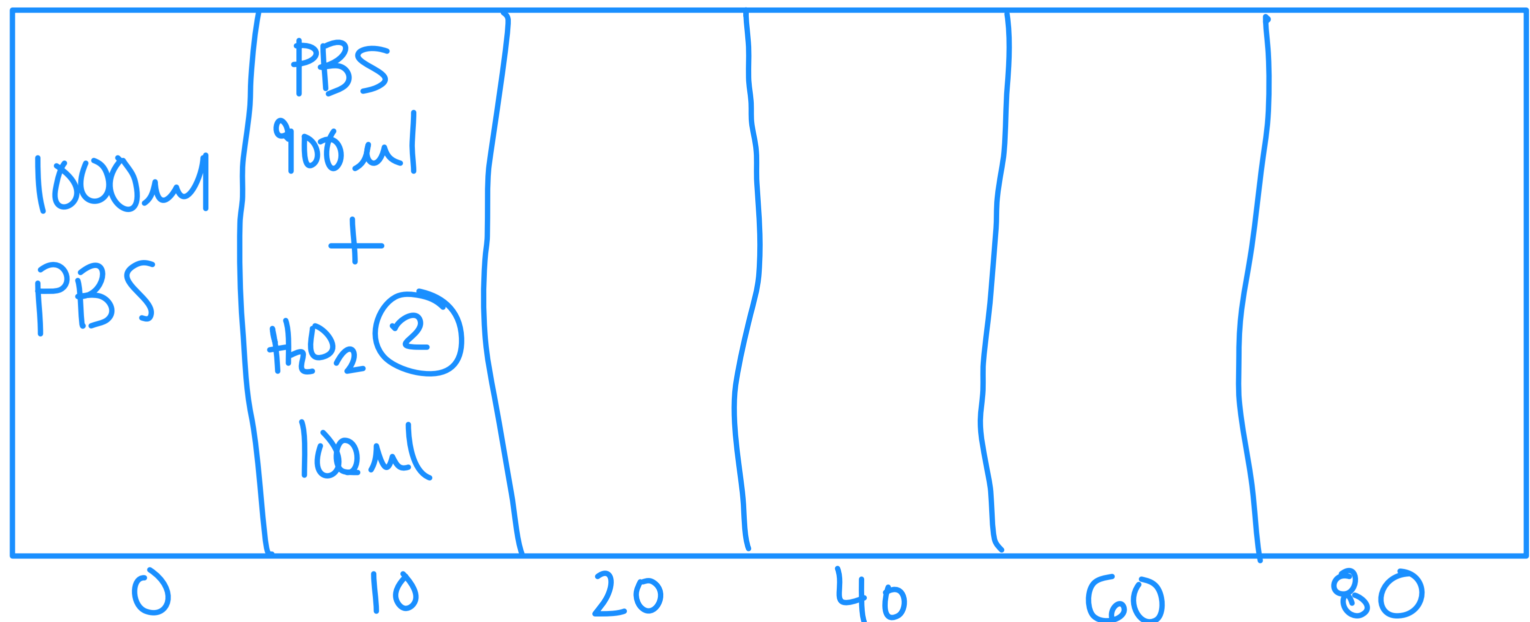
$C_1V_1 = C_2V_2$

Treat cells with 6 different doses of H_2O_2

- Stock H_2O_2 is 10M
- Add 100µl of drug dose to each macrowell
- Add drug to the "live" chip and "dead" chip in parallel
- Prepare dilutions in cold PBS
- Treat with : 0, 10, 20, 40, 60, 80 µM
- *Dilutions should minimize waste!*

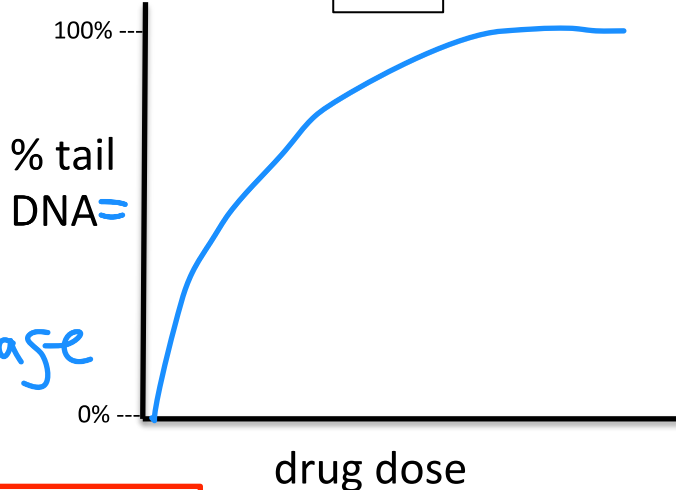
1) 10mL PBS + 10µl Stock H_2O_2 = 10mM
2) 10mL PBS + 100µl ① = 100µM

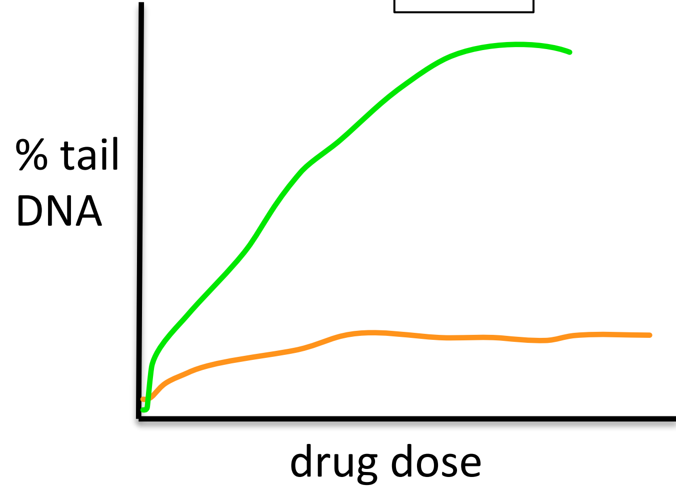
make 1mL (1000µl) of each dose



Why analyze dead cells?

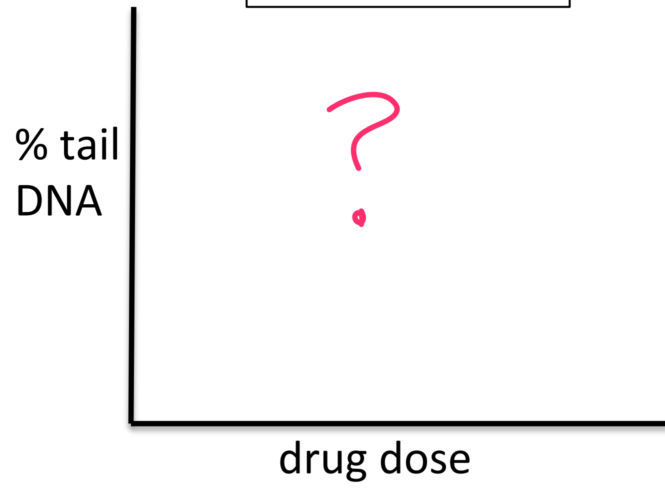
→enzyme dead





no tail=
no damage

tail=
DNA damage



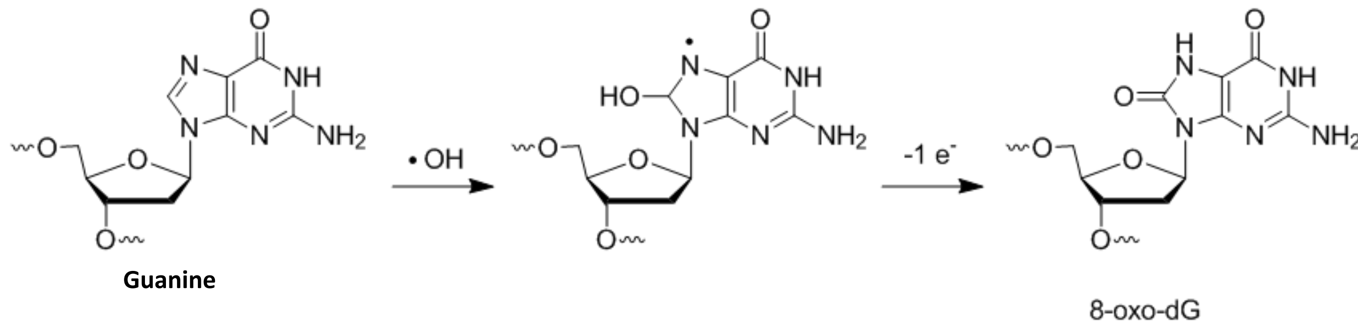
Note: W/F students will treat their TK6 cells with another drug, MMS.

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

How do H_2O_2 and MMS damage DNA?

oxidative damage

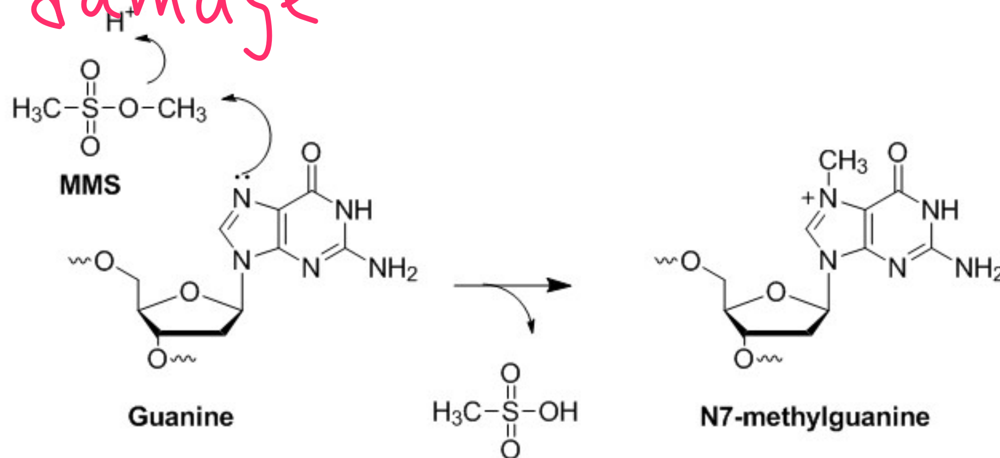
H_2O_2



Repaired by BER

alkylation damage

MMS



Lysis & electrophoresis of CometChips

- Alkaline lysis solution (> 1 h at 4°C)
  - 2.5 M NaCl, 100 mM Na_2EDTA, 10 mM Tris, (water)
  - pH 10
  - Triton X-100 detergent

  breaks open cells, denatures proteins

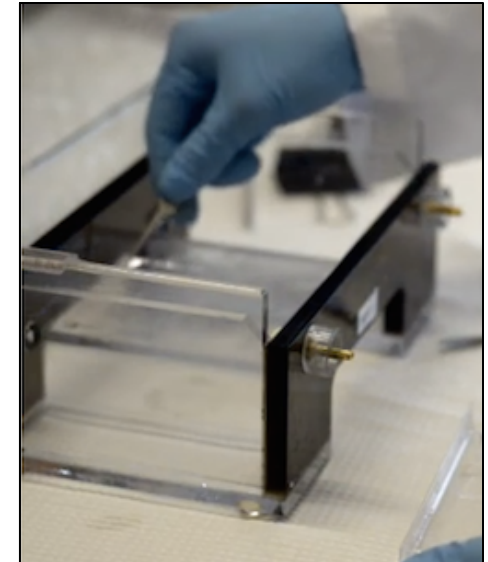
- Electrophoresis buffer (40 min incubation + 30 min run, at 4°C)
  - 0.3 M NaOH, 1 mM Na_2EDTA
  - pH 13.5 Very high pH
  - gellbond adhered with double-sided tape

  unwinds DNA

- Electrophoresis
  - 30 min
  - 1 V/cm (or 16 V for us)
  - 300 A

  very slow

- Neutralize & dye
  - Tris Base solution ~pH 7.4 (biological)
  - SYBR Gold → DNA stain



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 biology experience

Methods Practice

No 1 correct way to write a methods section!!

Maintenance of lymphoblast

~~Tissue Culture~~:

human lymphoblast cell line (gift of Engelward lab, MIT)

TK6 cells were grown in a ~~flask~~ with ~~12ml~~ RPMI

10% FBS, Pen Strep X units/mL

supplemented with FBS. The cells were kept in an

5% CO_2, 95% RH

incubator at 37°C. ~~A stain~~ was used to assess if the

Trypan Blue

cells were alive or dead.

Improving a Methods paragraph

Maintaining lymphoblastoid cell line(s):

- 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 (Invitrogen). All cell culture media were supplemented with 100 units/mL penicillin-streptomycin (Invitrogen).

average # cells per microwell

Yellow Team	
A1: 120, 000 cells, 40 min loading	2.13,3.02
A2: 120, 000 cells, 40 min	
B1: 30, 000 cells, 40 min	2.72,2.60
B2: 30, 000 cells, 20 min	

Green Team	
A1: 200,000 cells, 30min loading	2.40,1.70
A2: 200,000 cells, 30min loading	
B1: 150,000 cells, 30min loading	1.70
B2: 200,000 cells, 15min loading	

Blue Team	
A1: 100,000 cells, 35min loading	2.3,3.2,1.66
A2: 100,000 cells, 35min loading	
B1: 200,000 cells, 35min loading	
B2: 100,000 cells, 15min loading	1.57

Pink Team	
A1: 40,000 cells, 30min loading	1.6
A2: 75,000 cells, 30min loading	
B1: 75,000 cells, 30min loading	1.15
B2: 75,000 cells, 5min loading	

Purple Team	
A1: 150,000 cells, 20min loading	3ish
A2: 150,000 cells, 20min loading	
B1: 15,000 cells, 20min loading	
B2: 150,000 cells, 5min loading	

loaded dead @
~50K

lowest loading #s
30K
15K
40K

In lab today

1. Load TK6 to "live" chip:
   - 50,000 ________cells/well
   - For __25____min
   - during cell loading incubation prepare H_2O_2 dilutions on ice
2. Overlay with 1% LMP agarose
   - 3 min at room temperature + 3 min at 4 °C
3. Treat 2 CometChips with H_2O_2
   - one prepared by faculty, with "dead" (already lysed) cells
   - one prepared by you today, with live TK6 cells

Thesis Defense

Wednesday, September 21st (tomorrow!)

Time: 3:30 PM

Location: 56-614

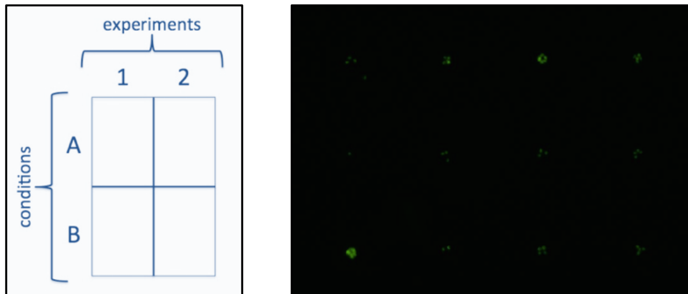
Investigation into the Role of DNA Damage and Repair during Influenza Infection and Inflammation

by Marcus C. Parrish

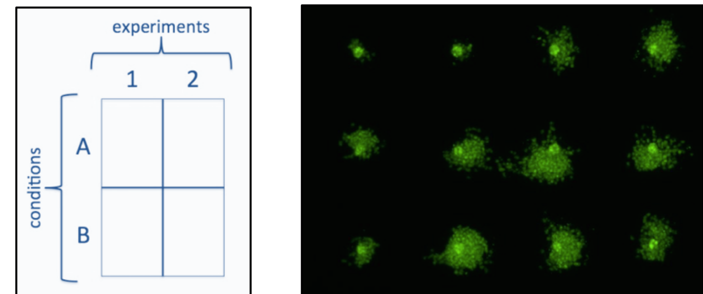
Engelward lab

M1D2: Analysis of Doubling Time Expt.

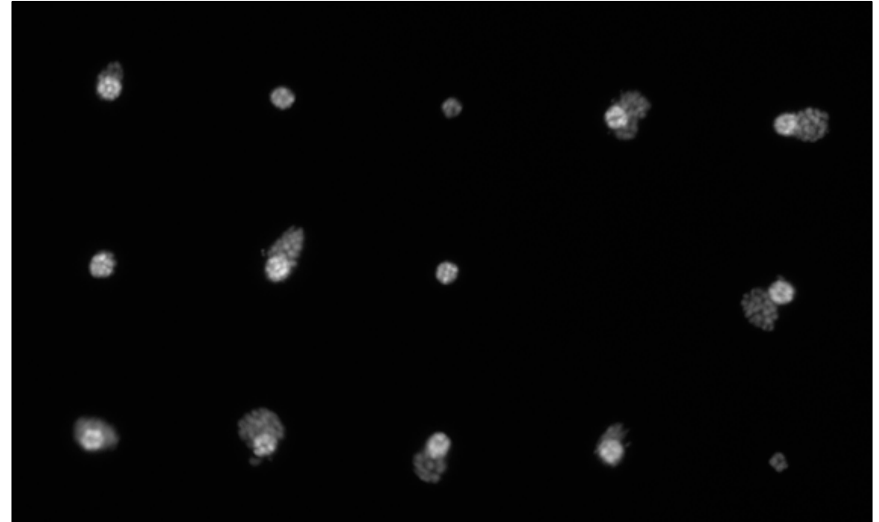
in main lab (T=0 days)



in TC room (T=~~4~~ 25 days)



Doubling time of TK6 cells – sample & images



- T = 0
- fixed overnight at 4 °C with 10% formalin (on Thurs 09/15 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 Sunday 09/18 at 10am)
- labeled overnight at 4 °C with Vybrant Dye Cycle Green (Life Tech.)
- (not lysed)

VERY sensitive DNA stain

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

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

- Published TK6 doubling time = 20 h