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Introduction to Fluorescence & Absorbance

Jacob Beal

iGEM Measurement Summer Webinar: Quantifying fluorescence and cell count with plate readers

July 14th, 2020

Fluorescent Protein = Debugging Output

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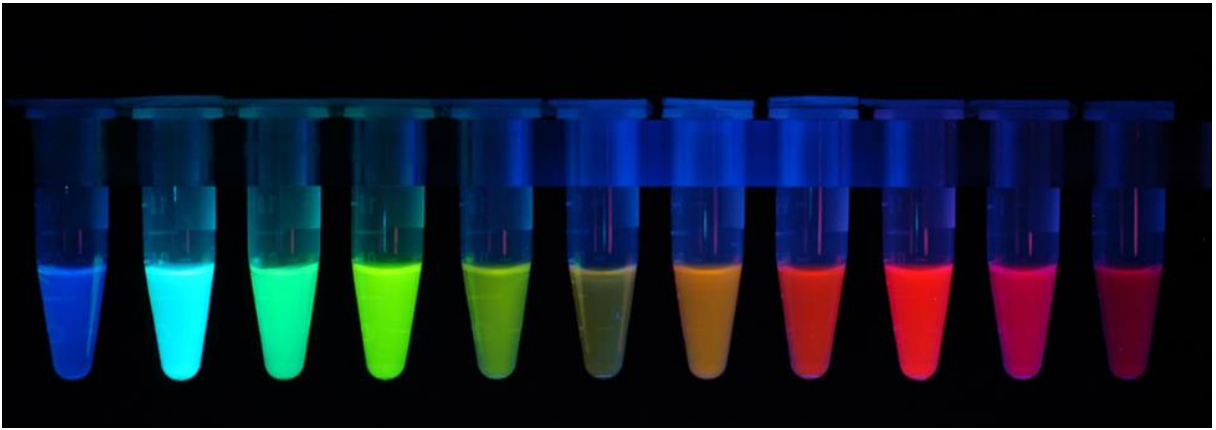
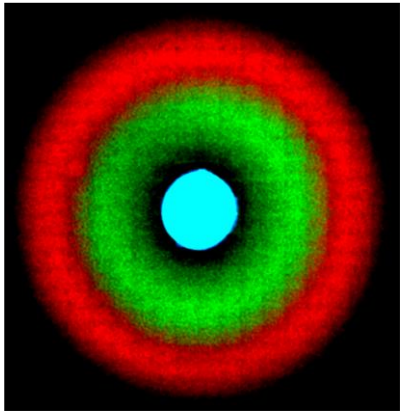
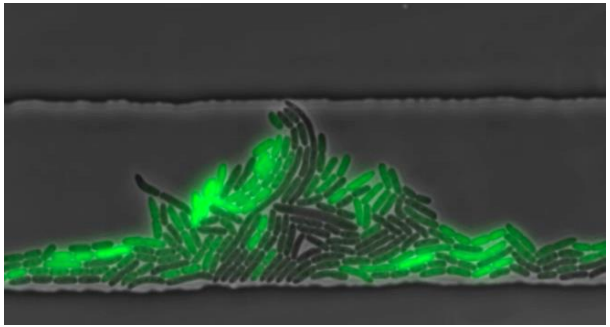


[Tsien Lab]



[Weiss]

Up to ~15 boolean, ~3-4 precision quantification



[Hasty]

Picking your instrument

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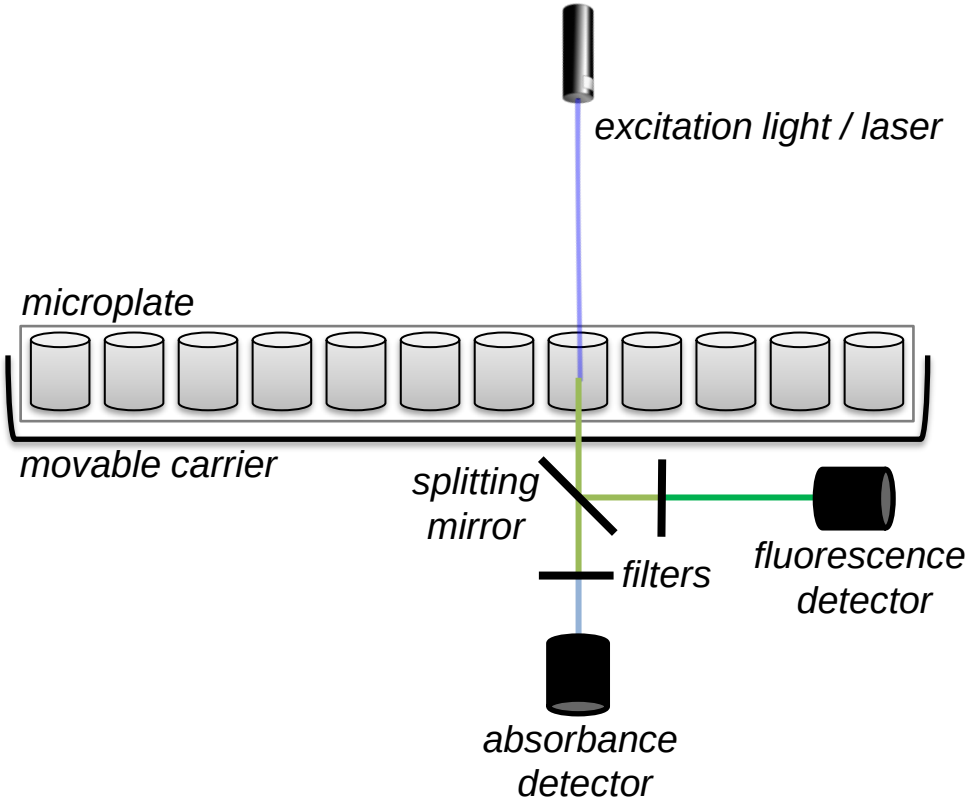
Today's focus

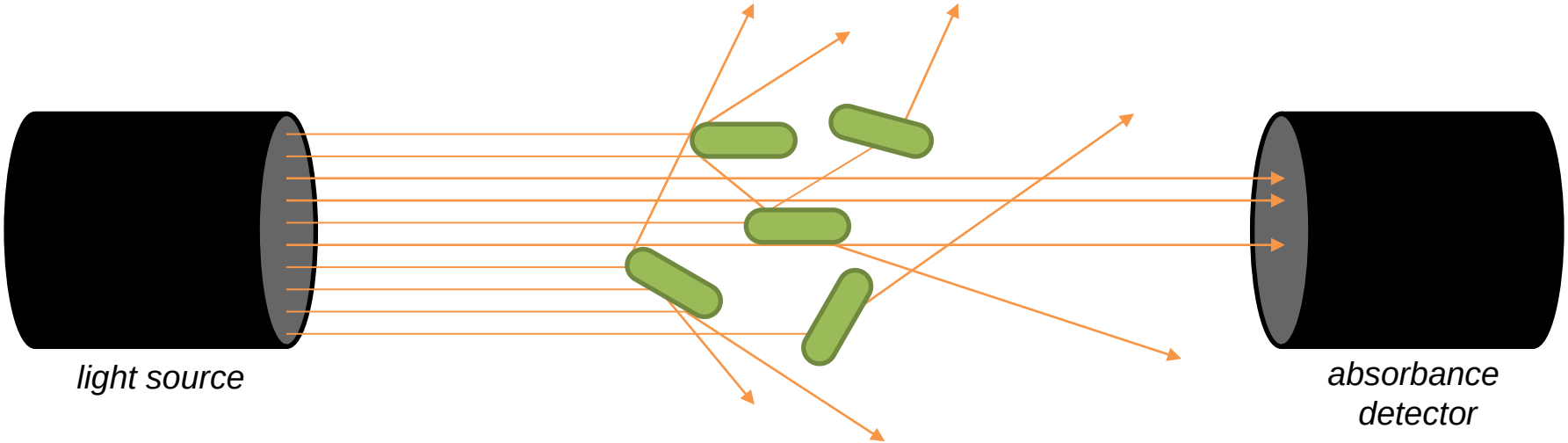
	Fluorimeter	Plate Reader	Flow Cytometer	Fluorescence Microscope
Throughput	Low	High	High	Low - High
Resolution	Population	Population	Single Cell	Subcellular
Time Series	Yes	Yes	No	Yes
Dynamic Range	2-3 logs	2-3 logs	3-6 logs	1-2 logs
# cells	n/a	n/a	High	Low

Of course, your resources and instructors matter most…

How a plate reader works

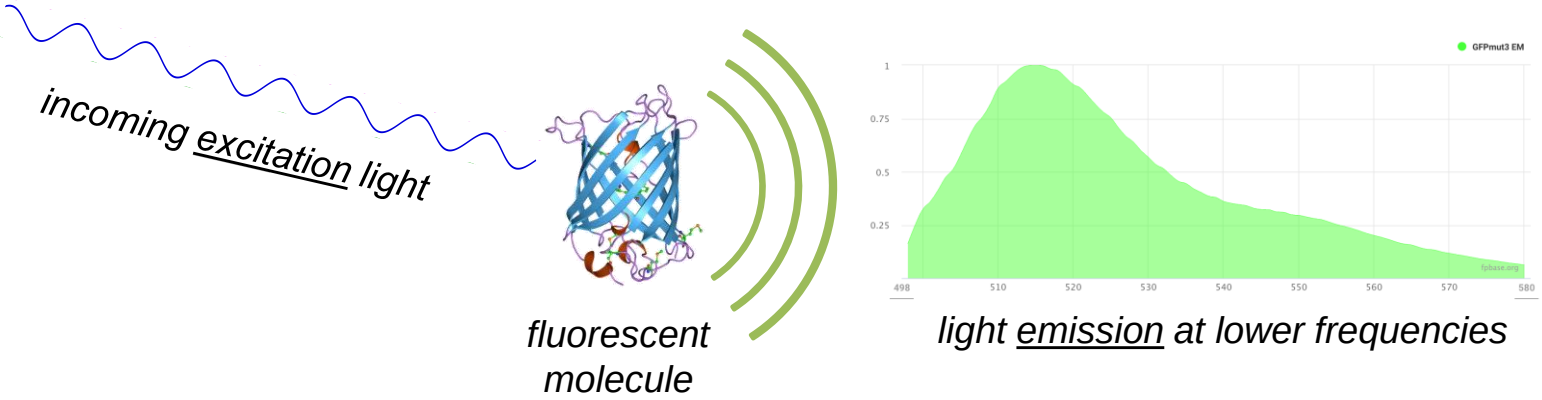


How Absorbance (OD) Works



- Cells (and other particles) scatter and absorb light
  - Optical Density (OD) = $\log_{10}$ (source / detector)
- Fraction of light detected depends on particle density, particle opacity, path length
  - Scattering also depends on frequency (typically 600nm)
- Absorbance per cell linear at low OD (< ~0.5), highly non-linear at high OD (> ~2.0)

How Fluorescence Works



- Great signal, proportional to number of molecules
- Exact unit relation in context is highly sensitive, hard to calculate

Factors affecting fluorescence measures

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- Molecule:
  - Excitation and emission spectrum
  - Brightness / quantum yield
  - Proper folding
  - Quenching from binding to other molecules
- Sample:
  - pH, oxygenation
  - Background fluorescence: media, cell (can vary with cell state)
  - Other fluorescent molecules (spectral overlap)
  - Sample volume, orientation
- Instrument
  - Excitation strength, frequency
  - Emission filters, light path
  - Overlap of excitation and emission
  - Detector amplification

Fluorescence always needs calibration to independent standards & process controls

Excitation and Emission Spectra

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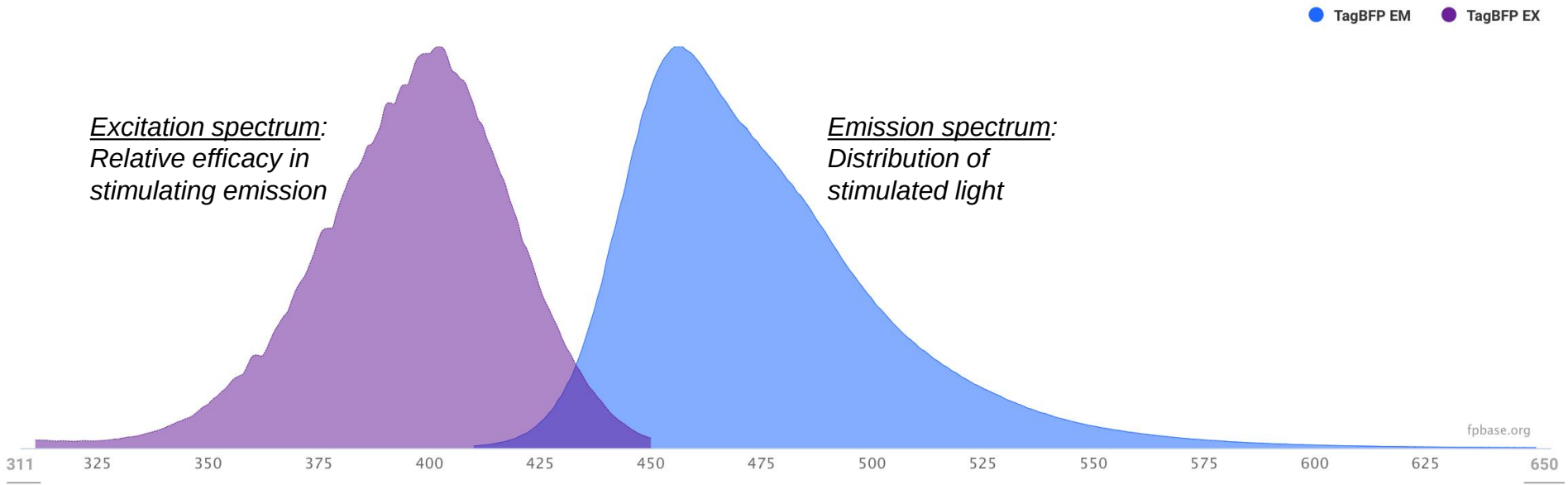
Excitation spectrum:
Relative efficacy in stimulating emission

Emission spectrum:
Distribution of stimulated light



Excitation and Emission Spectra

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- Spectra should match your instrument capabilities, not overlap significantly
- Minor overlap (recommend <3%) can be disentangled with a linear transform

Poorly Separated Excitation & Emission

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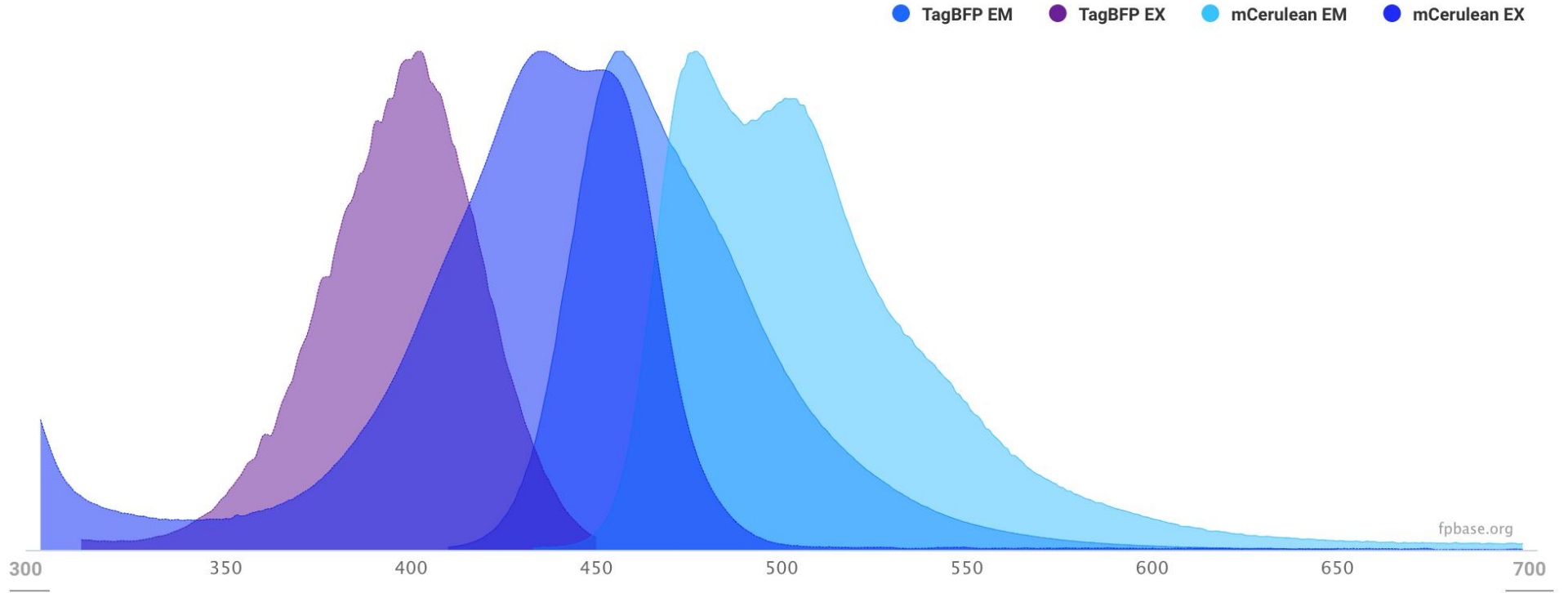

- TagBFP and mCerulean can be distinguished, but are too close for good quantification

Separated by Excitation

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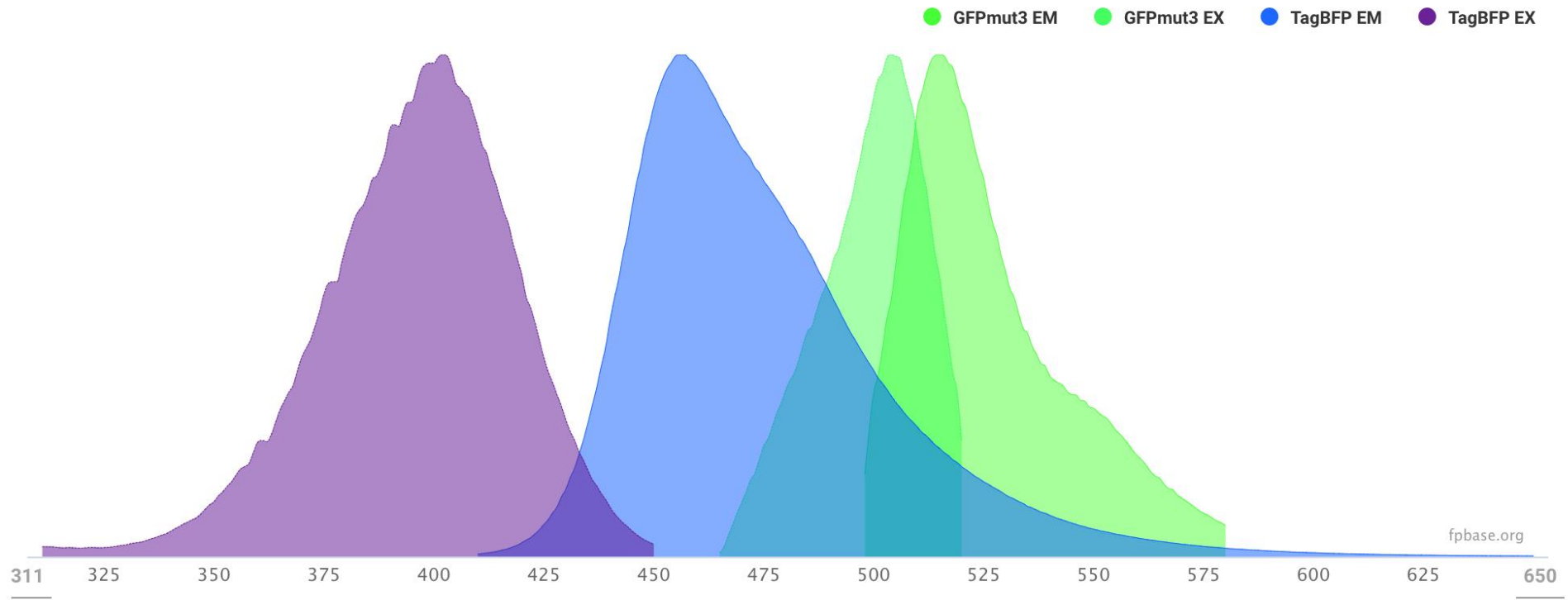
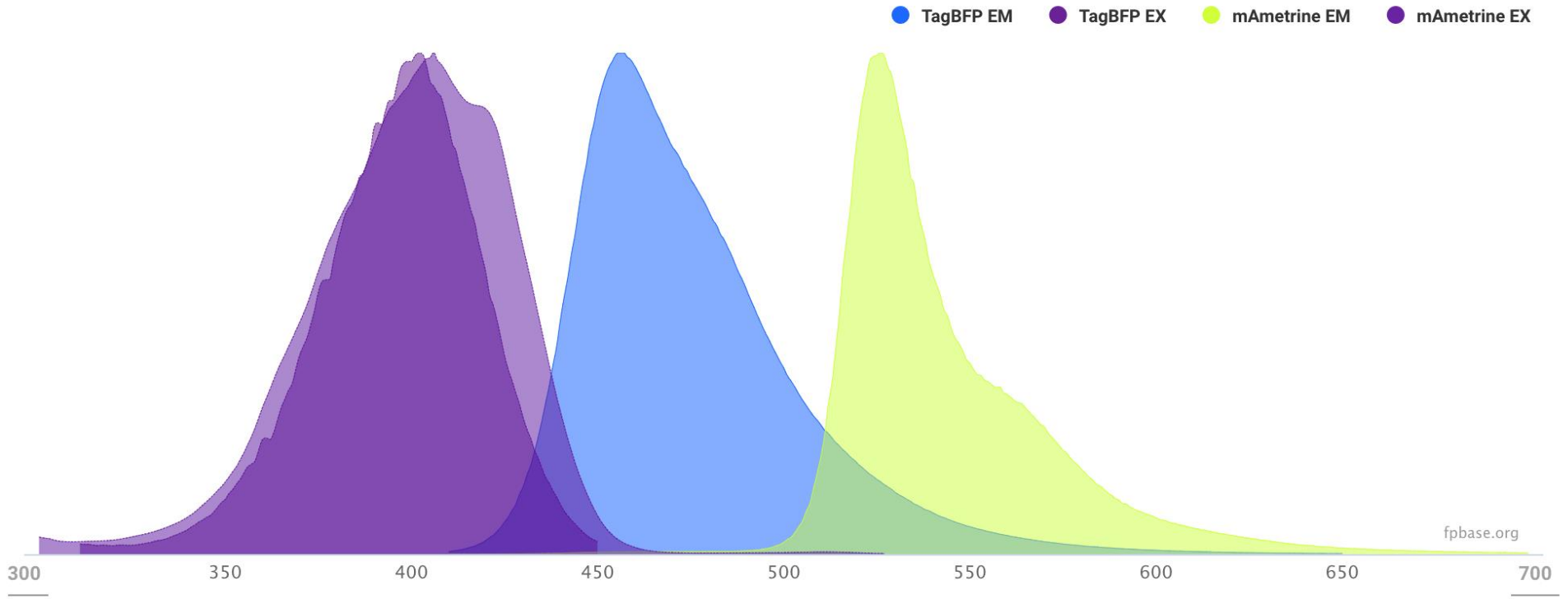


- TagBFP and GFPmut3 are excited by different wavelengths, thus do not overlap in readings

Separated by Emission



- TagBFP and mAmetrine are excited similarly, but emission is sufficiently separated.
- Useful if your instrument cannot do multiple independent excitations

Recommended Fluorescent Proteins

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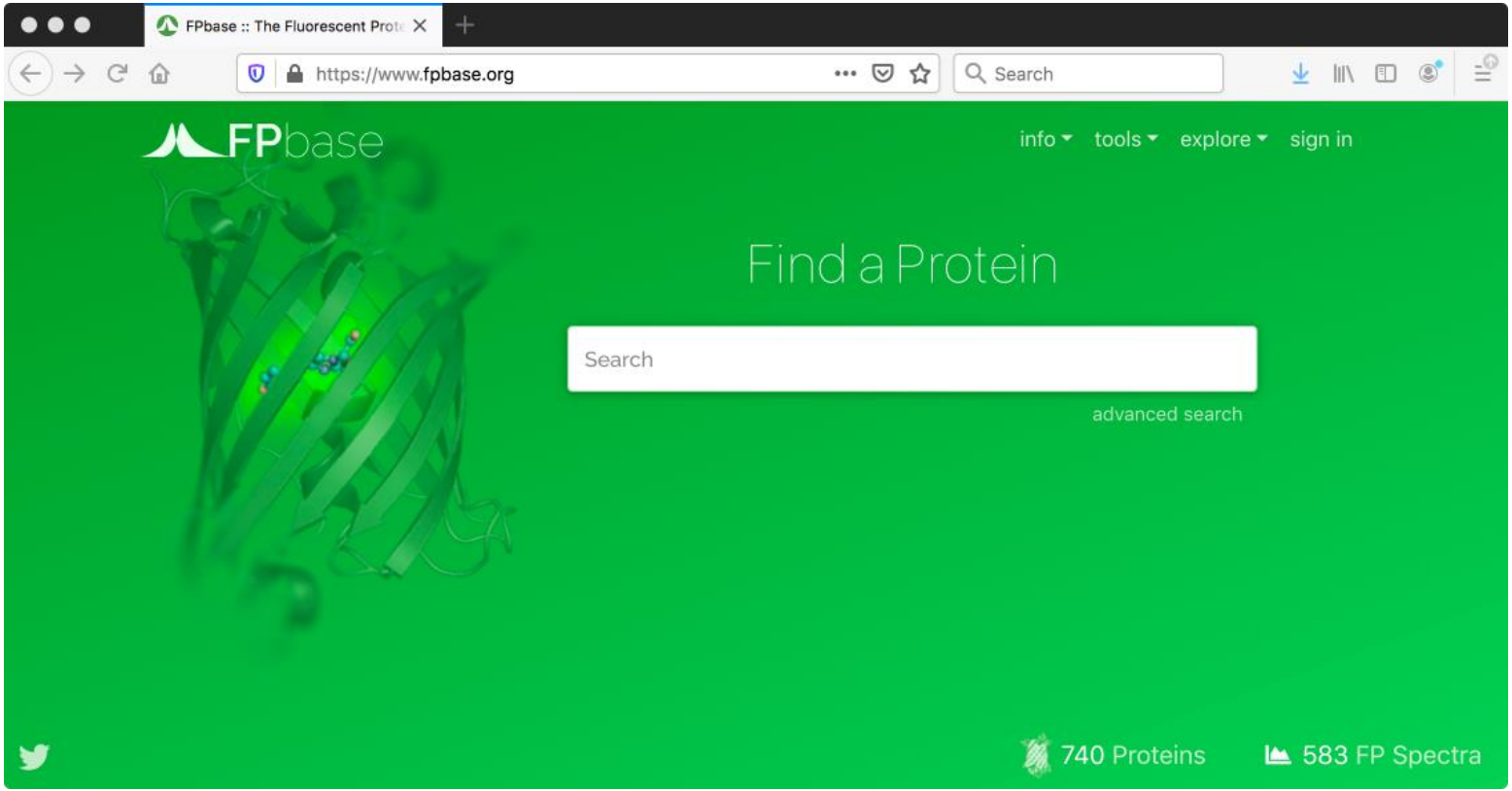
- **Green Fluorescent Proteins**
  - **BBa_E0040**: GFPmut3 (Excit. 500 / Emiss. 513, brightness 35, maturation time 4.1 min, weak dimer).
  - **BBa_K864100**: sYFP2 (Excit. 515 / Emiss. 527, brightness 68, maturation time 4.1 min).
  - Calibrant: fluorescein
- **Red Fluorescent Proteins**
  - **BBa_J06504**: mCherry (Excit. 587 / Emiss. 610, brightness 16, maturation time 15 min, pKa 4.5).
  - mKate2 (Excit. 588 / Emiss. 633, brightness 25, maturation time 20 min, pKa 5.4).
  - If a slow maturation time is acceptable:
    - **BBa_E1010**: mRFP1 (Excit. 584 / Emiss. 607, brightness 12.5, maturation time 60 min, pKa 4.5).
    - mScarlet (Excit. 569 / Emiss. 594, brightness 70, maturation time 174 min, pKa 5.3).
  - Calibrants: Texas Red, Nile Red
- **Blue Fluorescent Proteins**
  - **BBa_K592100**: TagBFP (Excit. 402 / Emiss. 457, brightness 33, maturation time 13 min, pKa 2.7).
  - If a slow maturation time is acceptable:
    - Cerulean3 (Excit. 433 / Emiss. 475, brightness 35, maturation time 70 min, pKa 3.2).
  - Calibrants: Coumarin 30 *(not yet verified)*

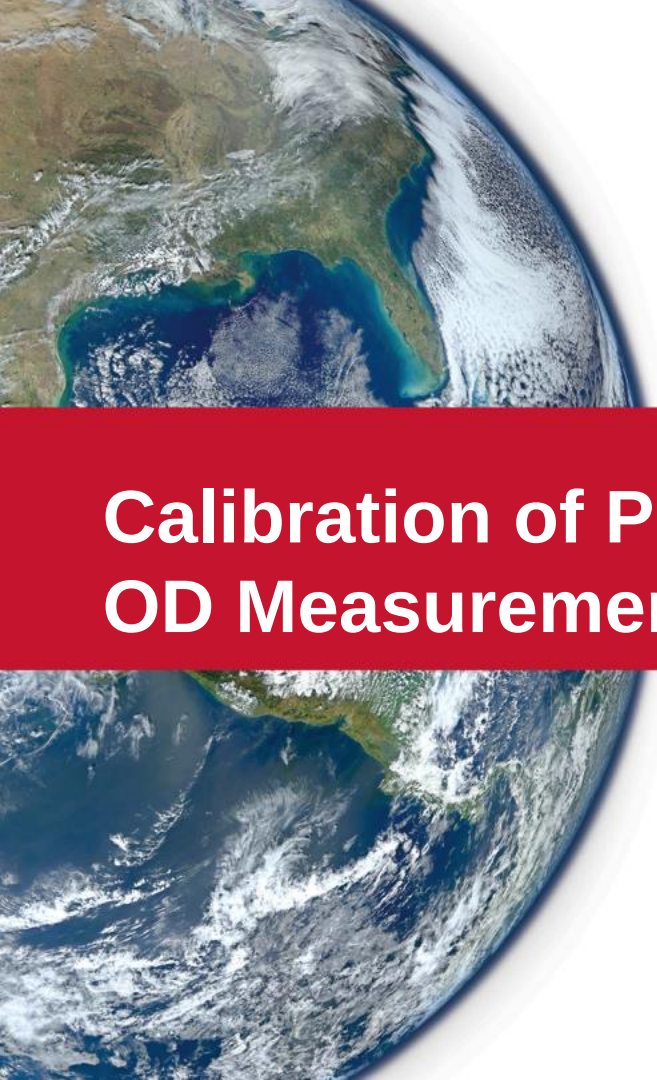
If you need other proteins:



For more proteins & spectra, fpbase.org is an excellent resource!

Summary

- Fluorescence is a valuable biological debugging tool
- Different instruments are good for different purposes
  - Plate readers are good for tracing time series
  - Flow cytometers are good for quantifying cell behavior
  - Microscopes are good for spatial arrangement and subcellular structure
- Plate readers typically measure both absorbance (OD) and fluorescence
- Fluorescence is affected by many factors, so measurements must be calibrated
- Fluorescent proteins should be chosen to match calibrants and not interfere with each other



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Calibration of Plate Reader Fluorescence and OD Measurements

Jacob Beal

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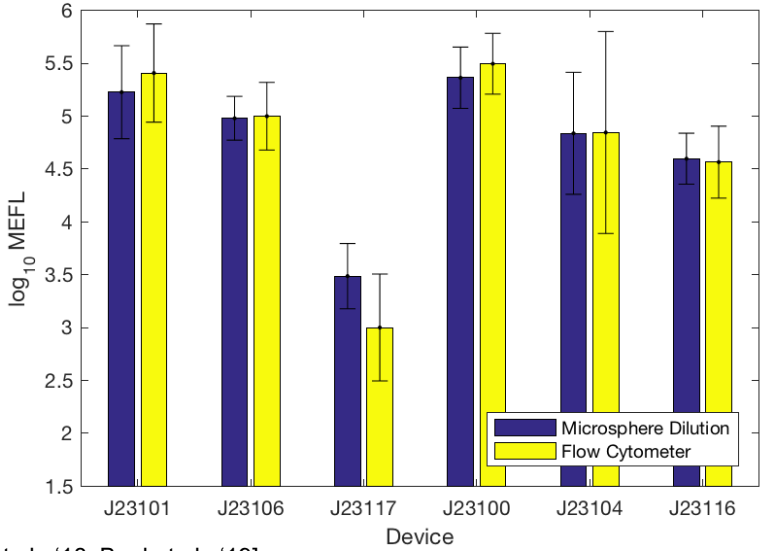
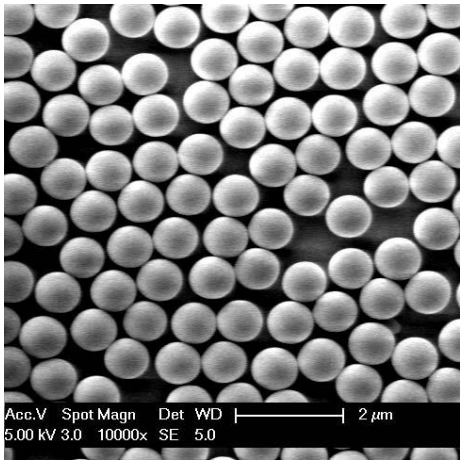
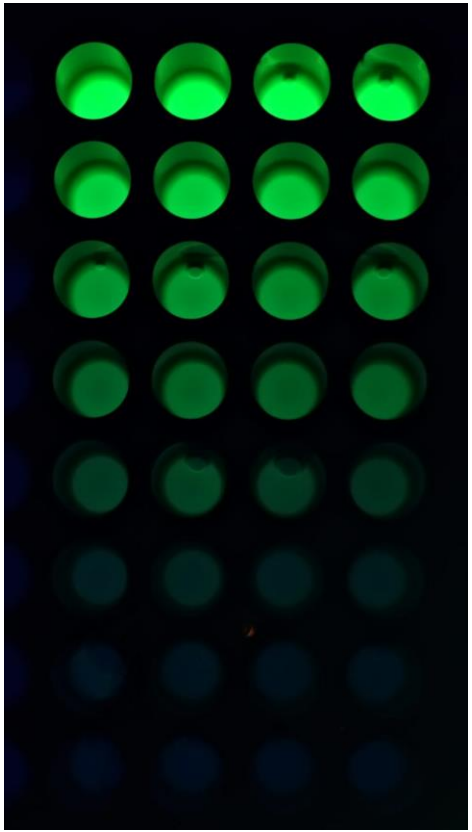
July 14th, 2020

iGEM Plate Reader Calibration Protocol

- Calibration with serial dilutions of cheap, stable materials
  - GFP: fluorescein (Sigma 46970), RFP: Texas Red (Sigma S3388)
  - OD: Monodisperse silica beads (Nanocym 950nm)
- Produces MEFL units directly comparable w. flow cytometry, models
- Highly replicable & debuggable measurements (1.8x geo.std.)
- Validated with large-scale interlab study (244 institutions)







[Beal et al., '18, Beal et al., '19]

Calibration Process

- Running the calibration protocol: https://2020.igem.org/Measurement/Protocols
  - Serial dilution of monodisperse silica particles
  - Serial dilution of each fluorescent dye
  - Enter all values in provided Excel sheet to calculate
- Instrument settings must be identical for calibration and experiment!
  - Turn off instrument auto-calibration! (e.g., path length correction, auto-gain)
- Re-run calibration at least monthly (preferably weekly), to ensure nothing changes

Serial Dilutions

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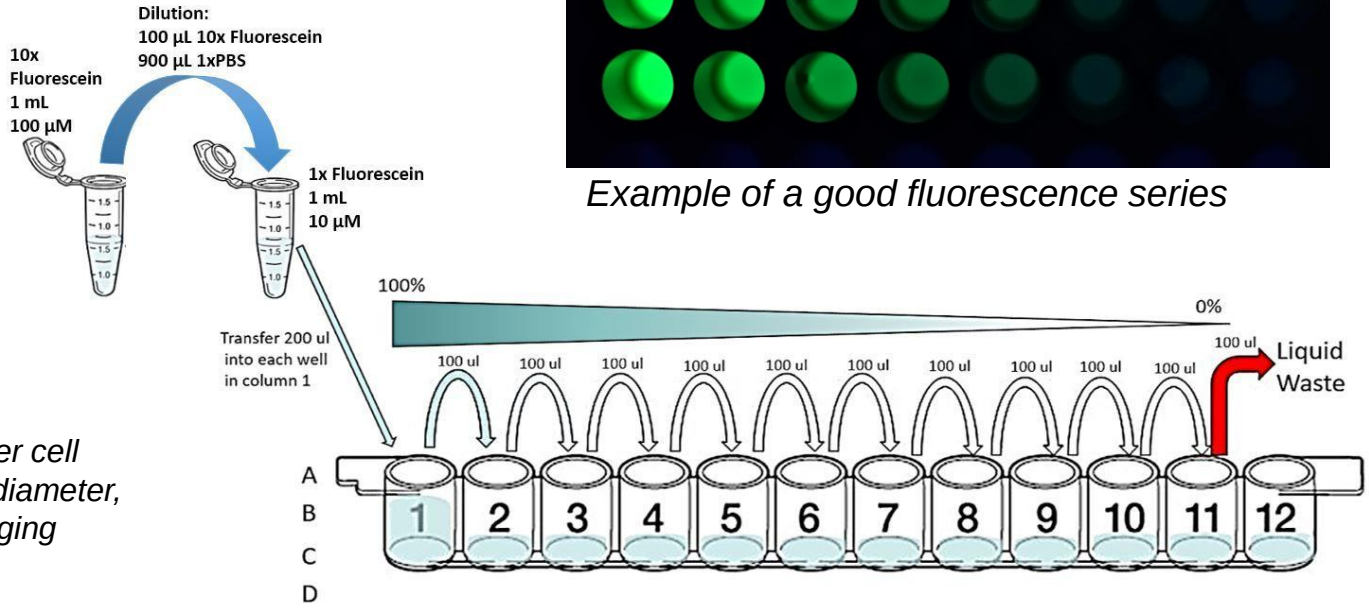
Stock concentrations:

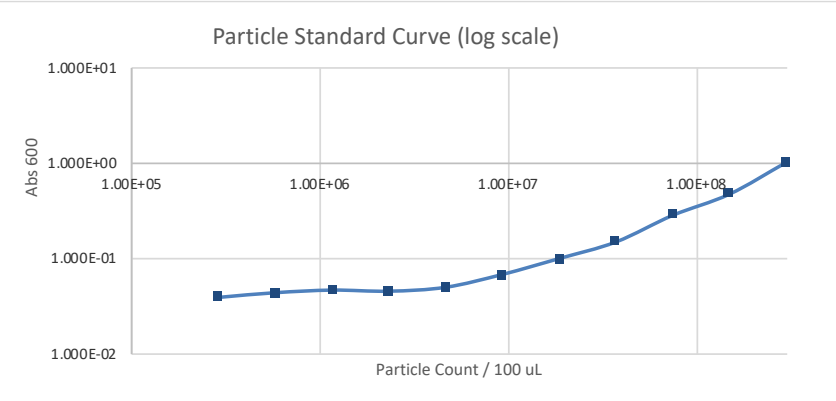
- OD: 3.00E+09 Particles / mL
- Fluorescein: 10 µM
- Texas Red: 2 µM

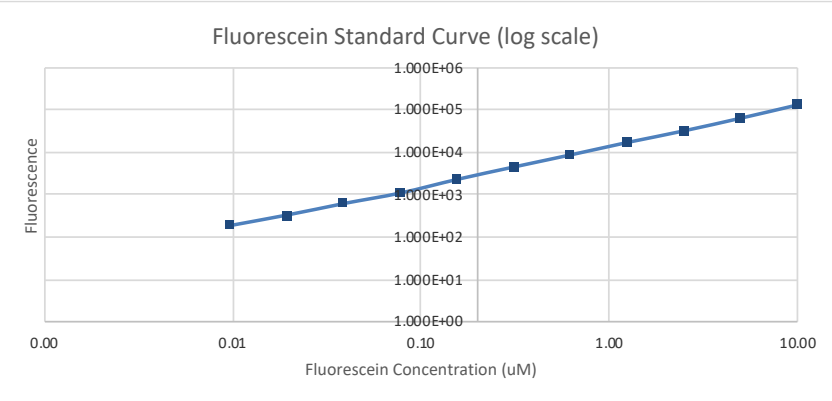
Example of a good fluorescence series



Should be adaptable to other cell types by changing particle diameter, other fluorescence by changing calibrating dye

Example of Good Calibration



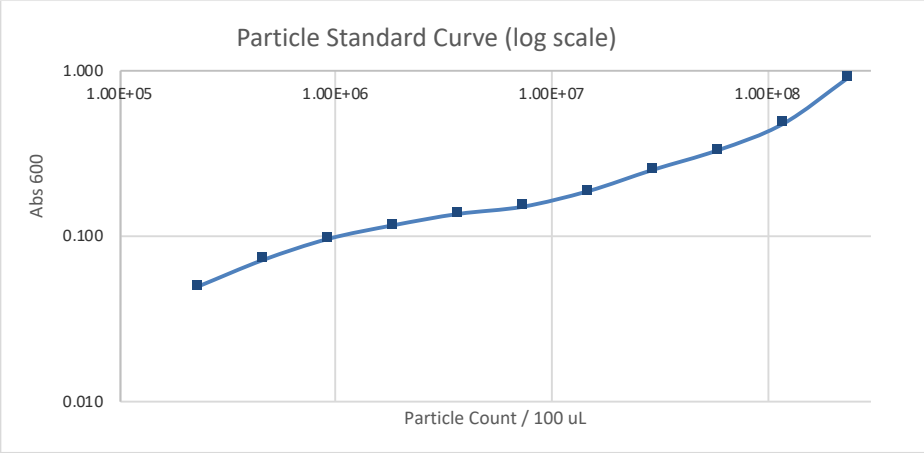


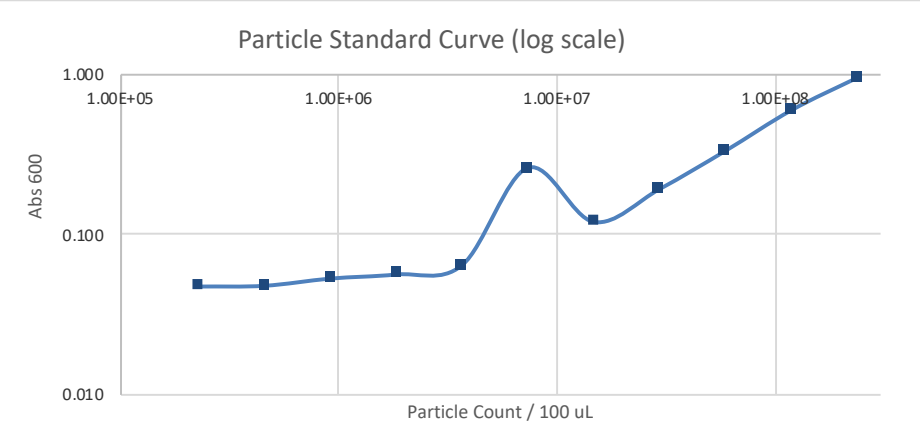
3.26E+08 particles / Abs600
Valid for Abs600 > 0.043

4.70E+09 MEFL / a.u.
Valid for a.u. > 47

For most instruments, effective dynamic range of fluorescence is larger than for OD

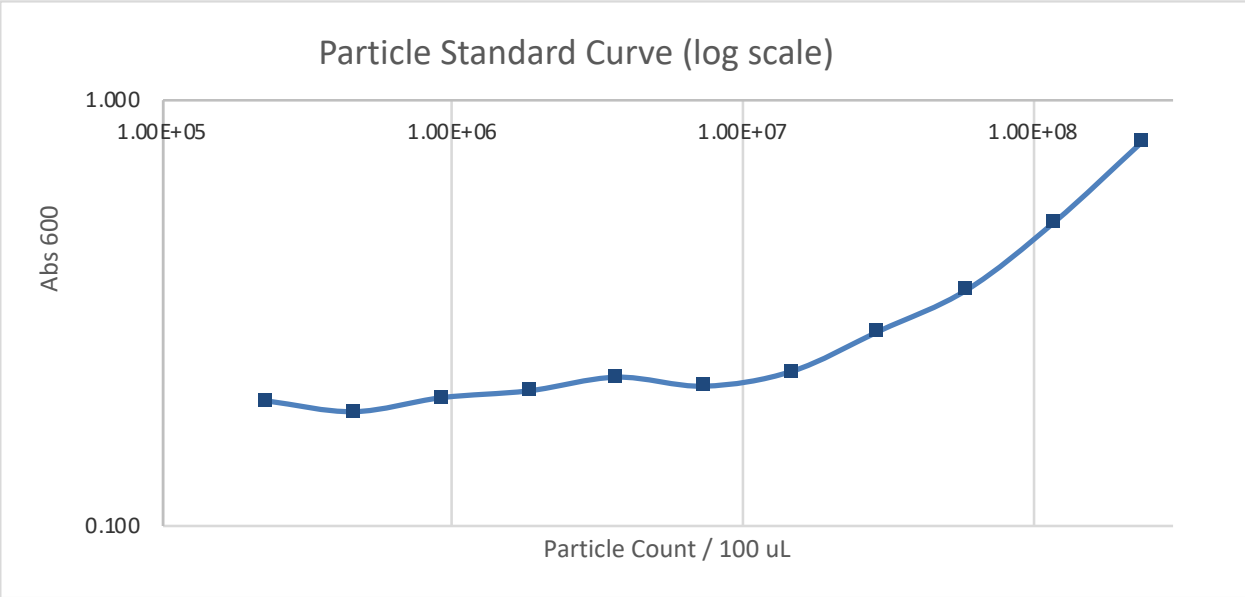
Problem: Inconsistent Dilution





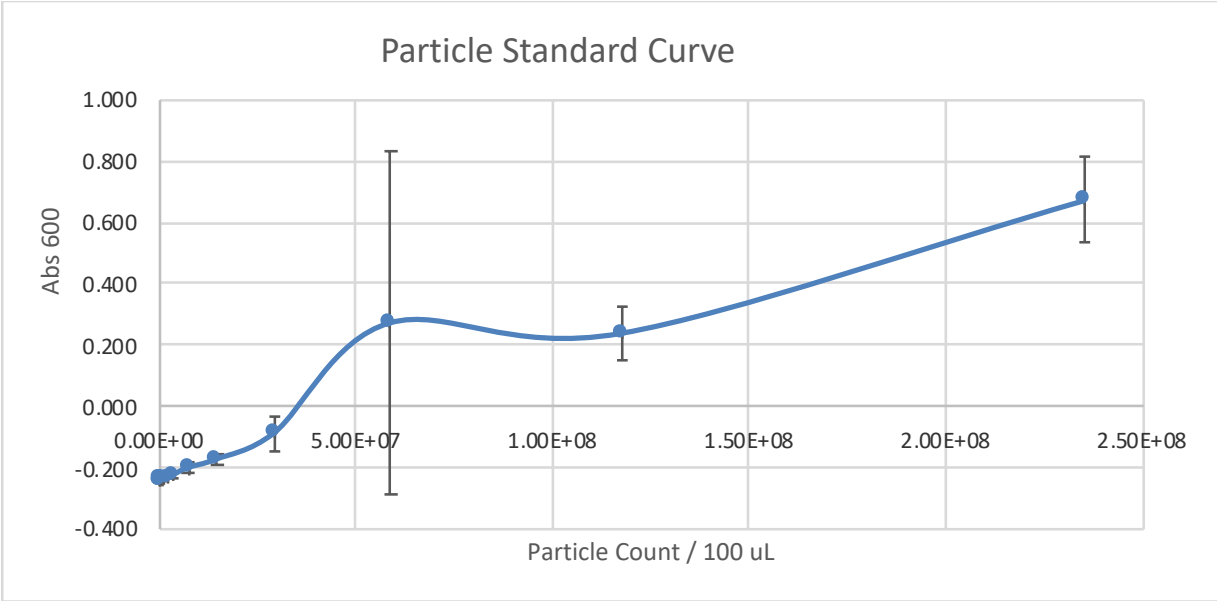
- If not linear in log scale (except for saturation), check data entry and/or redo pipetting

Problem: Narrow Range



- Only about 4x from highest to lowest range → adjust instrument settings
  - OD range should be at least 20x
  - Fluorescence range should generally be larger, depending on machine

Problem: Negative Values



- Readings should all be positive → instrument needs maintenance / reconfiguration

Automated Calibration Validation

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https://github.com/iGEM-Measurement-Tools/Excel_Process_Validator

```
Found Excel file iGEM 2019 Plate Reader Fluorescence Calibration - Example.xlsx
All expected sheets are present
Template appears to be intact
All variables were extracted
Sufficient dynamic range of Abs600 calibration values: 31.46
Found a sufficiently long particle dilution slope from column 1 to 7
Computed mean particles / Abs600 is positive
All non-blank wells show significant cell counts
All validation checks passed for Abs600
Sufficient dynamic range of fluorescein calibration values: 4387.20
Found a sufficiently long fluorescein dilution slope from column 1 to 11
Computed mean MEFL / a.u. is positive
All validation checks passed for fluorescence
```

What do the units mean?

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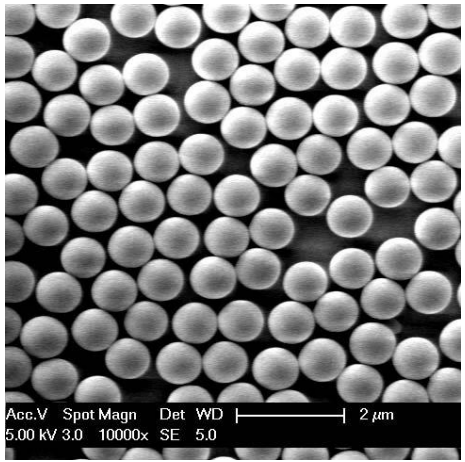
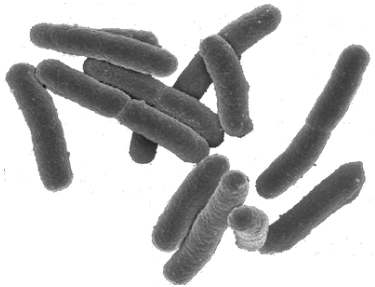
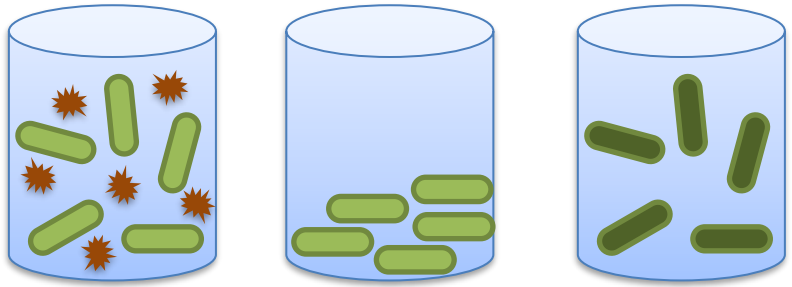
- Equivalent Particles
  - Calibrated units are close to cell counts (but not exact)
  - Measure can be increased by debris, packing, expression of opaque materials



E. coli ≠ silica spheres





"This sample is as opaque as a suspension of this many cell-like spheres"

What do the units mean?

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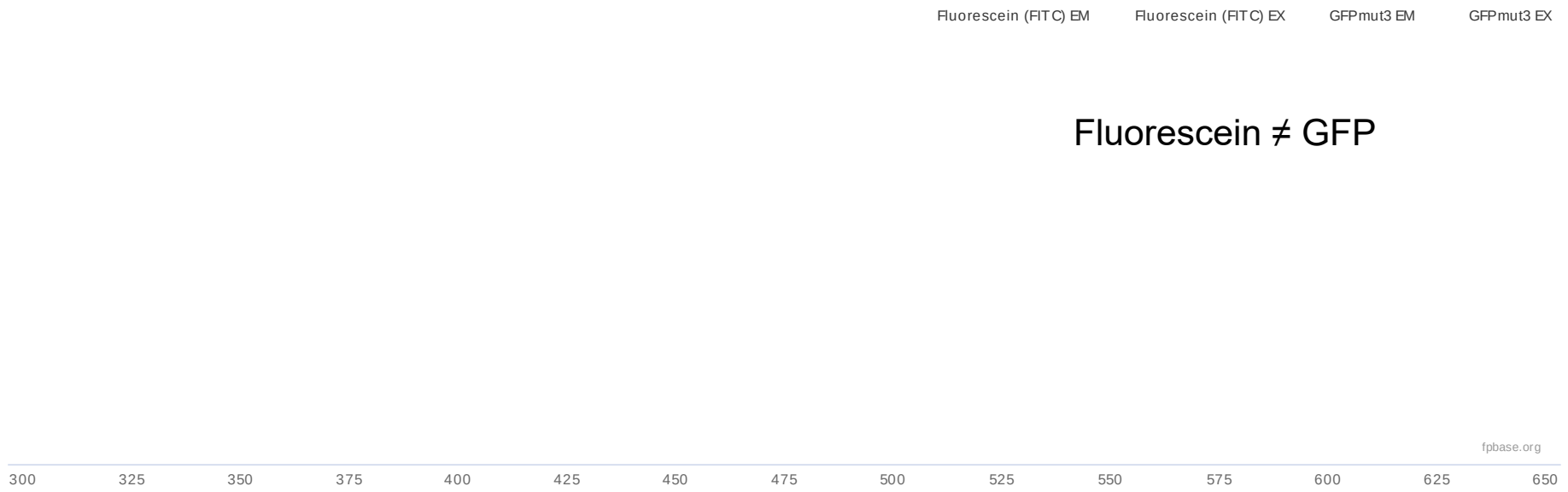
- Molecules of Equivalent X (e.g., MEFL X = Fluorescein, METR X = Texas Red)
  - Calibrated units are close to molecule counts (but not exact)
  - Measure be decreased by fluorescence inhibitors (e.g., slow folding, lack of oxygen)



Fluorescein ≠ GFP

"This sample is as fluorescent as this many molecules of fluorescein"

Summary

- Plate reader calibration is simple, cheap, and reliable
  - Use consistent settings and recalibrate at least monthly
- Calibration can detect problems with instrument or configuration
  - Automated validation software is provided by iGEM
- Calibrated units are close to cell count & molecule count (but not identical)
  - Some types of conditions can cause large value changes

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Interpreting & Debugging Plate Reader Data

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July 14th, 2020

Estimating cell and molecule counts

- Raw readings include background, even after calibration
- To get accurate estimates, subtract background:

$$Cells = (OD - media) * \frac{particles}{OD}$$

$$\frac{MEFL}{Cell} = (a.u. - WT\ a.u.) * \frac{MEFL}{a.u.} * Cells$$

Calibration Excel templates do cell estimate and MEFL conversion, but not WT subtraction

Gene expression → geometric statistics

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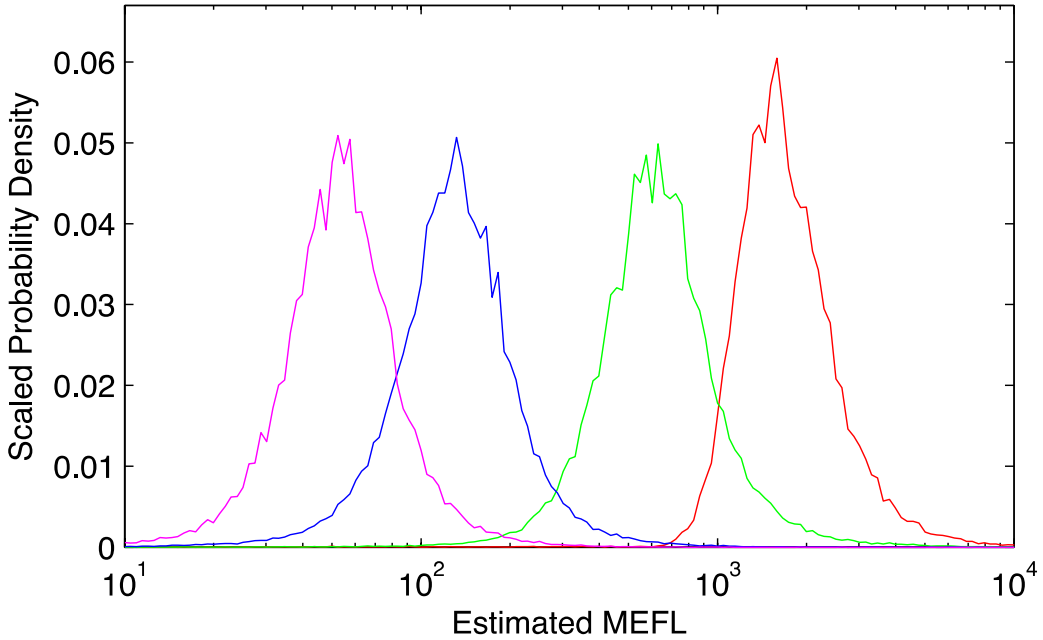
- Use geometric statistics in analysis:



- Strongest fraction may dominate population

Why geometric stats?

Complex catalytic reactions
→ multiply many rates:

$$R_{express} = R_1 R_2 R_3 R_4 R_5 \ldots$$

Central Limit Theorem
→ converge to log-normal!

Gamma distribution bursting also implies geometric stats

[Davidsohn et al., 2015], [Beal, 2017]

How to Compute Geometric Statistics

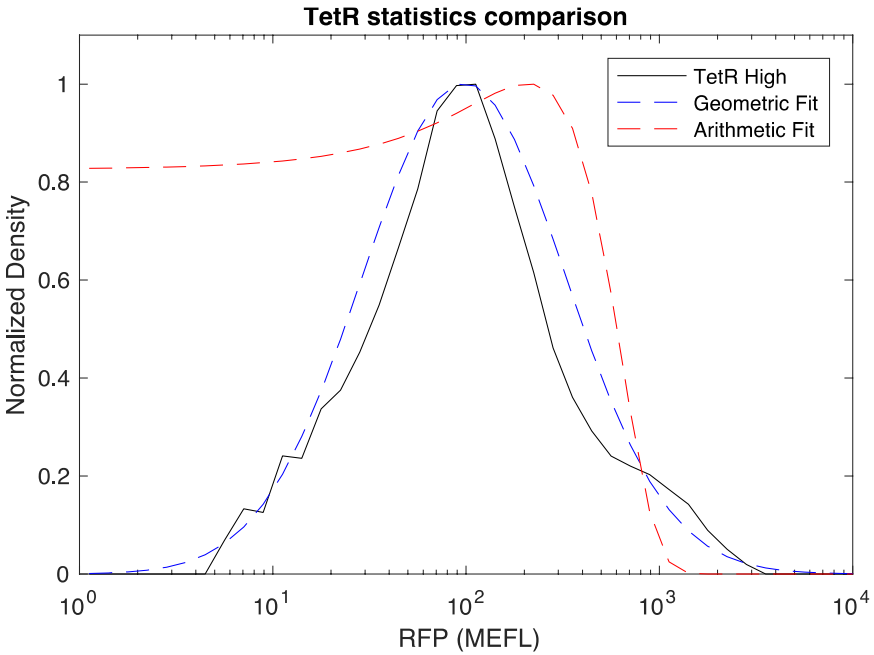
Geometric statistics are just normal (arithmetic) statistics on a logarithmic scale:

- Geometric mean = 10^(mean(log10(data)))
- Geometric std.dev. = 10^(std(log10(data)))

Consequences:

- Error bars no longer “plus/minus”
- Instead: k-fold “times/divide”

Example of geometric vs. arithmetic statistics on per-cell fluorescence data:



What about zero?

- Net absorbance, fluorescence may be at or below zero
  - Invalid on the log scale!
- Really this is just giving an instrument limit:

```
% Values close to autofluorescence / media indistinguishable from background
% geometric, because cells dominate
autofluorescence_std = geostd(negative_control_replicates);
indistinguishable_MEFL = autofluorescence_mean*(autofluorescence_std^2-1);
% arithmetic, because instrument error dominates
media_std = std(media_replicates);
indistinguishable_cells = 2*media_std;
```

- Interpret as “< value” rather than zero (e.g., “MEFL/cell < 470”, “cells < 1.3e6”)

Experimental vs. Process Controls

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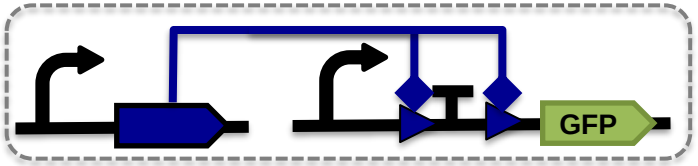
Experimental Controls:

- Is my hypothesis true?
- One control per factor under study
- Best when new data
- Control very close to experiment conditions

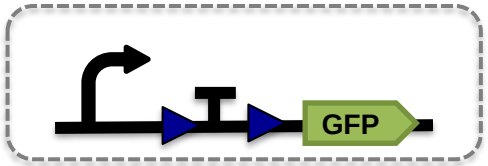
Process Controls:

- Should I trust the data?
- One control per assumption in study
- Best when known value
- Control should have minimal relation to experiment conditions

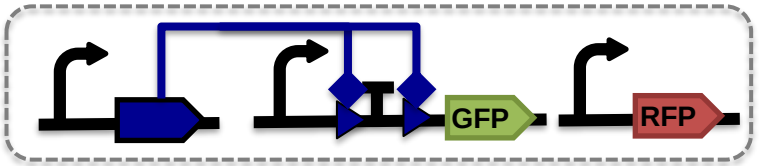
Experimental vs. Process Controls

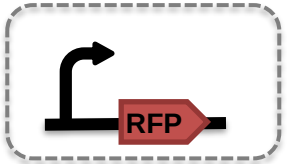


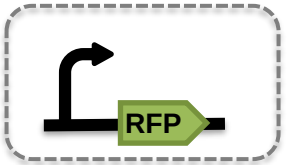
Experimental Controls:



Process Controls:







wild type cells
ERF beads
media only
focusing fluid

Example Data to Analyze

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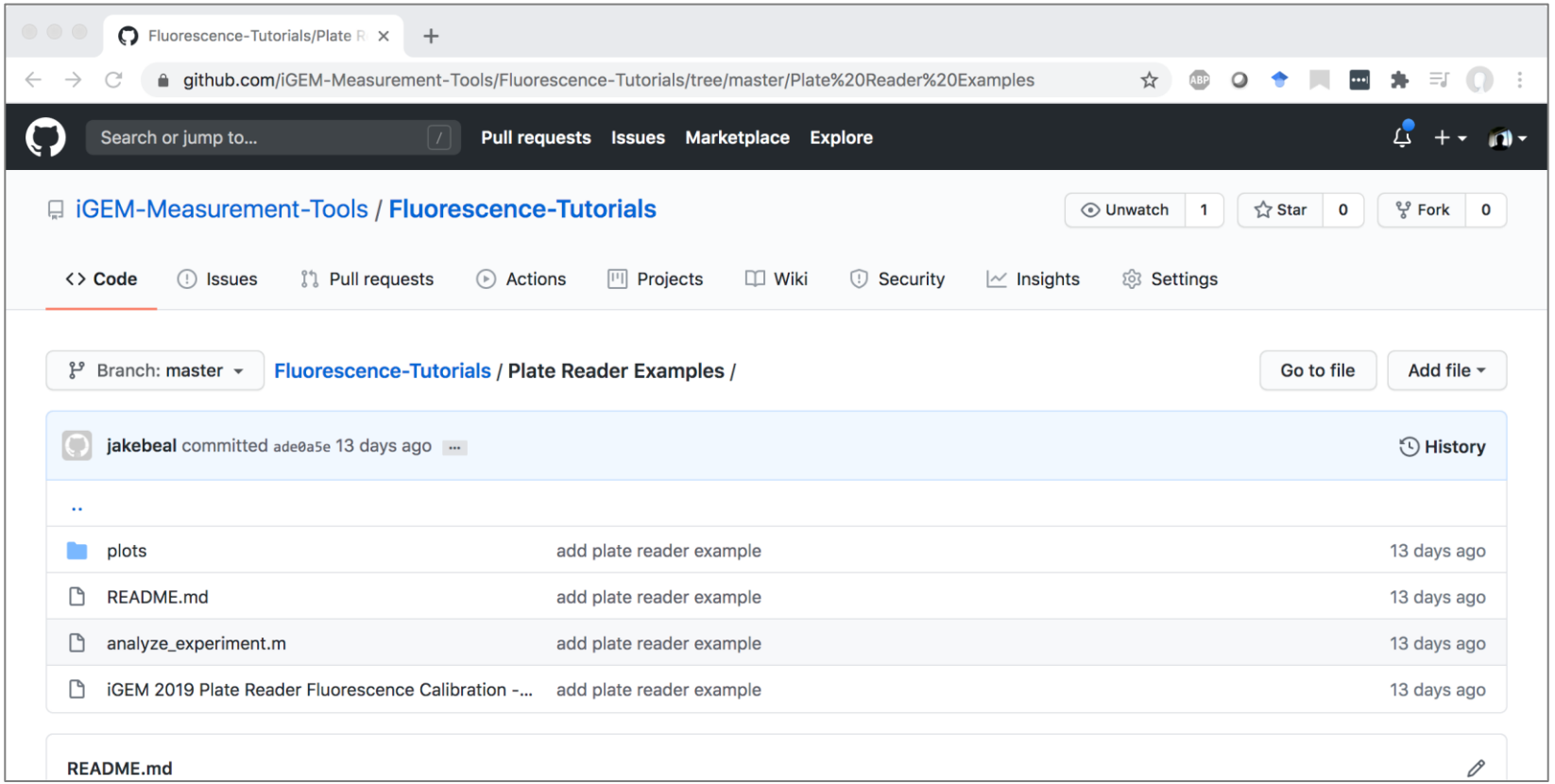
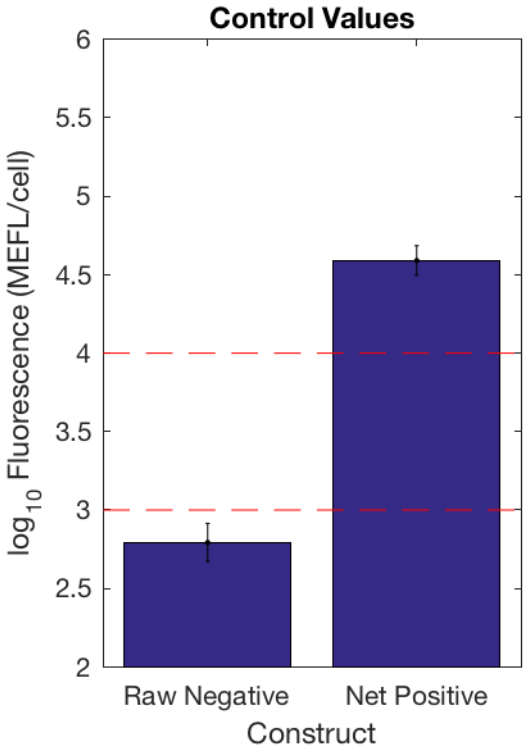


https://github.com/iGEM-Measurement-Tools/Fluorescence-Tutorials

Sanity Check Control Values

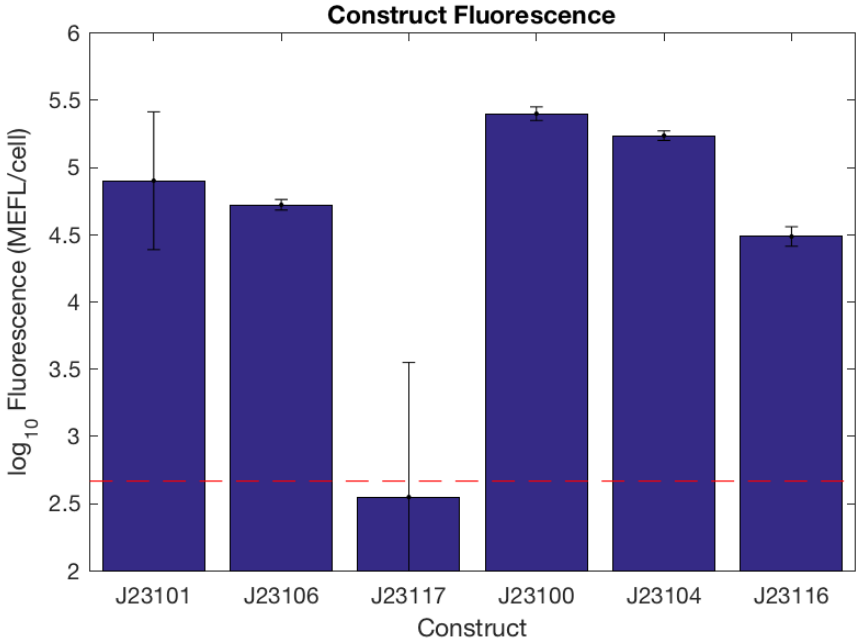
- Compare to calibrants to ensure instrument linear range
- Compare positive to max number of proteins per cell:
  - E. coli: 2e6
  - Yeast: 6e7
  - Human: 2e9
- Negative control should be much smaller than positive
- Problems with these values indicate likely process failure

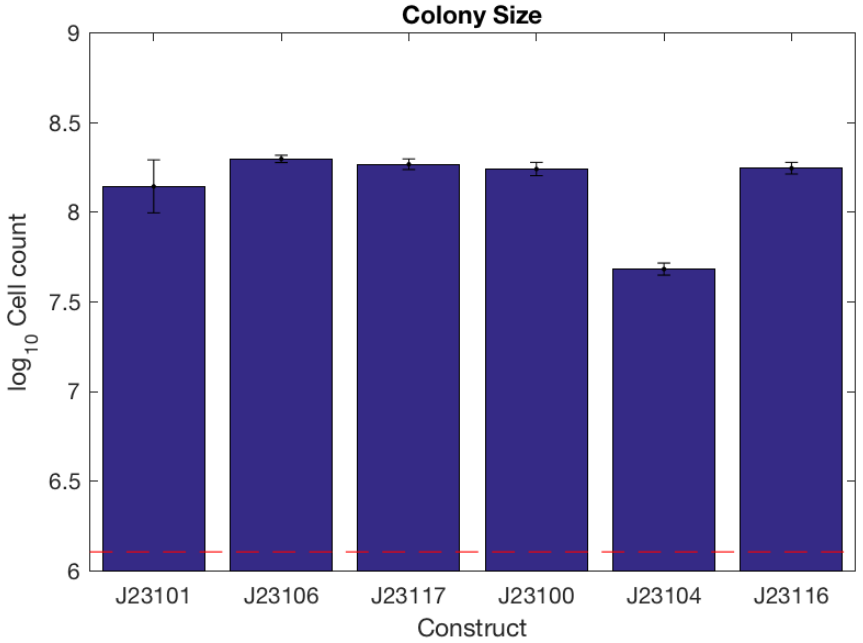
Example: E. coli negative <1e3, positive >1e4



http://book.bionumbers.org/how-many-proteins-are-in-a-cell/

Example of Experimental Data





- Colonies have generally grown well
- Constructs are covering full reasonable range of gene expression, except medium-low

Summary

- Calibrated units can be converted to estimates of cell / molecule count
- Gene expression should be analyzed using geometric statistics
- Low values are indistinguishable from background
- Data can be interpreted in relation to realistic biological values
  - Positive and negative controls can detect process failures
  - Experimental values can be related to biological intuitions