Flow cytometry: a powerful tool for biological investigations

Module 1, Lecture 7

20.109 Fall 2014

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Content adapted from Bevin Engelward

Topics for M1D7 Lecture

- Flow cytometry (FC)
  - why FC is awesome
  - how FC machine works
  - FC settings and analysis
    → focus on our experiment
- Module 1 in review, briefly
  - lab techniques + principles
  - scientific concepts

I’m not the only one who thinks so

Stanford Report, June 14, 2006

Kyoto Prize awarded to inventor of cell sorter

Herzenberg cobbled together the first FACS for $14,000 and dubbed it the 'Whizzer'

BY KRISTA CONGER

A search for life on Mars, the first ink-jet printer and nuclear weapons testing seem unlikely inspirations for a machine that changed the face of science and medicine. But to hear developer Leonard Herzenberg tell it, it all makes perfect sense. The Stanford researcher's feat of improbable alchemy, as well as his strong commitment to share his scientific and social accomplishments with others, has garnered him a 2006 Kyoto Prize, Japan's equivalent of the Nobel Prize.

Steve Gladfelter/VAS

Flow cytometry (FC) in a nutshell

- *What?* Evaluate cell fluorescence
  - one at a time
  - multiple channels
- *How?* Lasers plus printer technology
- *Why?* Let's you do lots of cool stuff

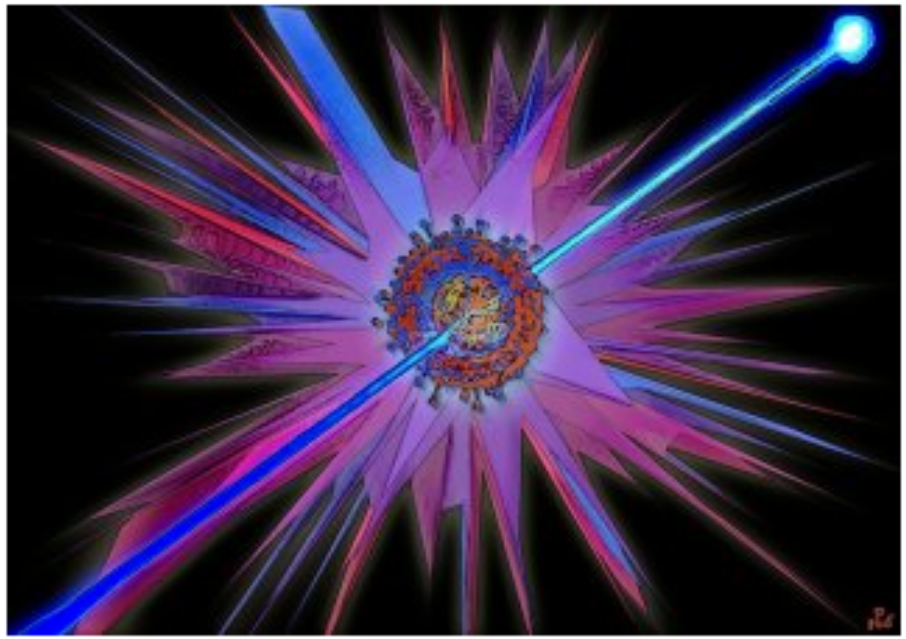
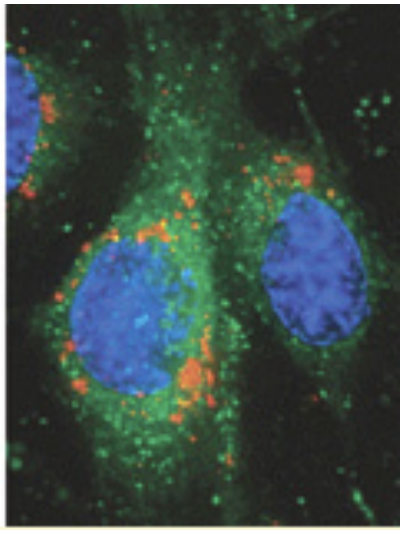


Image site: notproperlydone.com
Illustrator: Dr. Neil Peter Dufton

USES OF FLOW CYTOMETRY

Partner to FC: fluorescent molecules

- Antibodies conjugated to fluorophores
  - bind specific cell receptors
  - broad or narrow expression
- Genetically encoded fluorescent reporters
  - broad or under type-specific promoter
- Labeled small molecules
  - e.g., phalloidin binds actin
- Labeled molecules for uptake
  - nanoparticles



http://nano.cancer.gov/action/news/featurestories/monthly_feature_2005_dec.asp

FC cell type analysis: by scatter

- Distinguish blood cell populations
- Classic use of simplest FC
- Scatter = no fluorescence
- Just size/shape

High concentration (green) suggests a discrete population (its Gaussian peak)

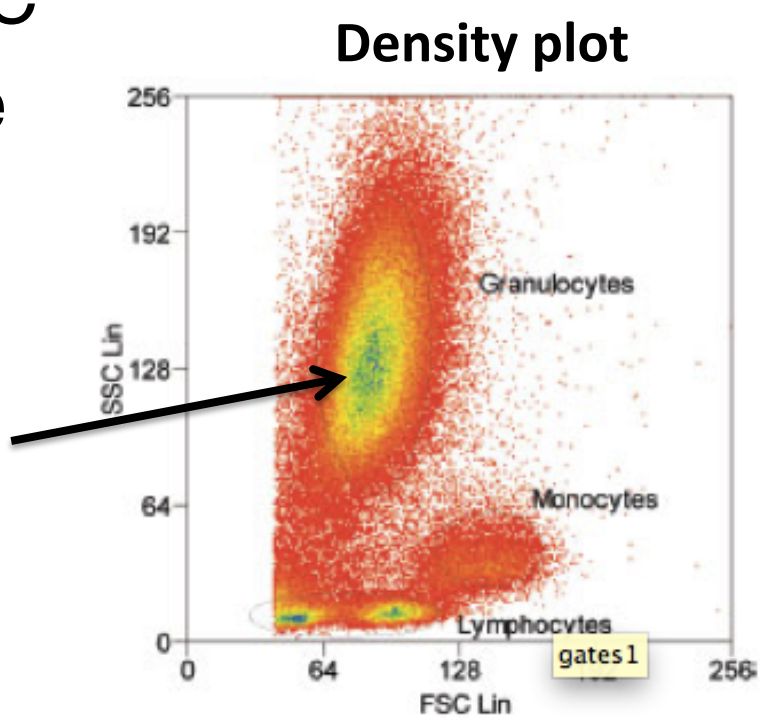


Image: http://www.abdserotec.com/flow-cytometry-gates-regions.html

FC cell type analysis: by fluors

- Assess different T cell populations w/ antibodies
- First select CD4 cells

- **Naïve: CD44^{lo}CD62L^{hi}**
- Memory: CD44^{hi}CD62L^{hi}
- Effector: CD44^{hi}CD62L^{lo}

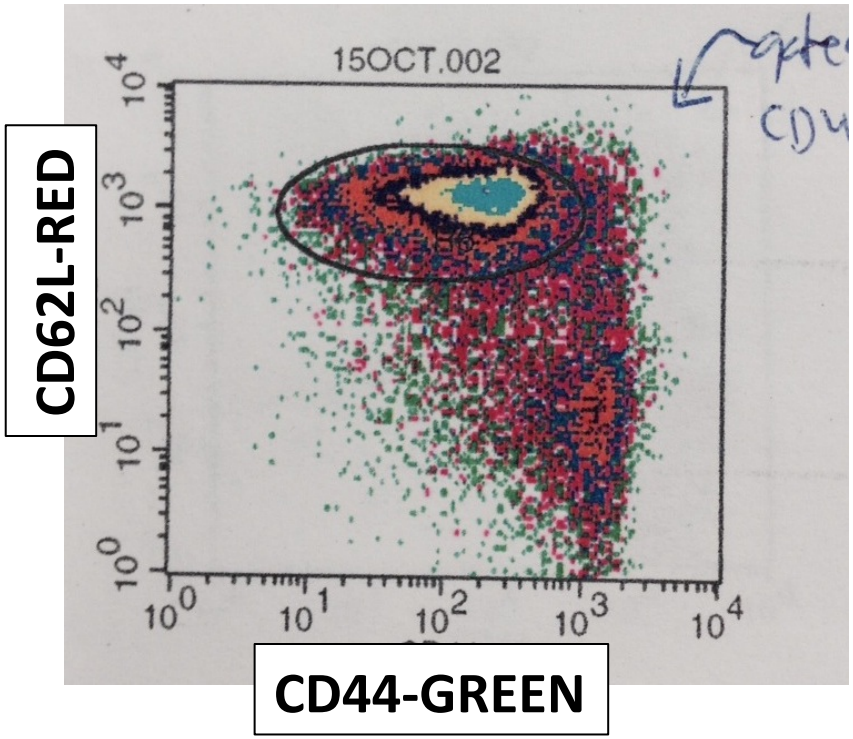


Image: A. Stachowiak

FC analysis of cell proliferation

- Small molecule dye
- Cytoplasmic
- Stain cells broadly
- Carried over during cell division
- [Dye] halved repeatedly

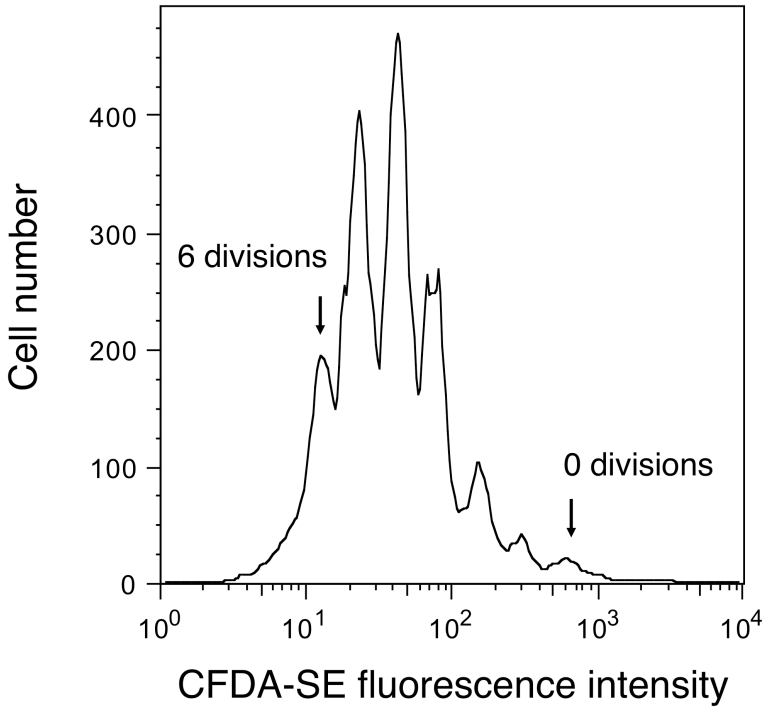


Image: A. Stachowiak

FC analyses of other cell functions

- Apoptosis
  - DNA intercalators, membrane im/permeable
  - antibodies to apoptotic program proteins (caspases)
  - other cell change detectors (e.g., calcium)
- Cell Cycle
  - quantify DNA by intercalator
  - plus BrdU uptake (T-analogue)
  - potentially multiple time-points
  - other variations

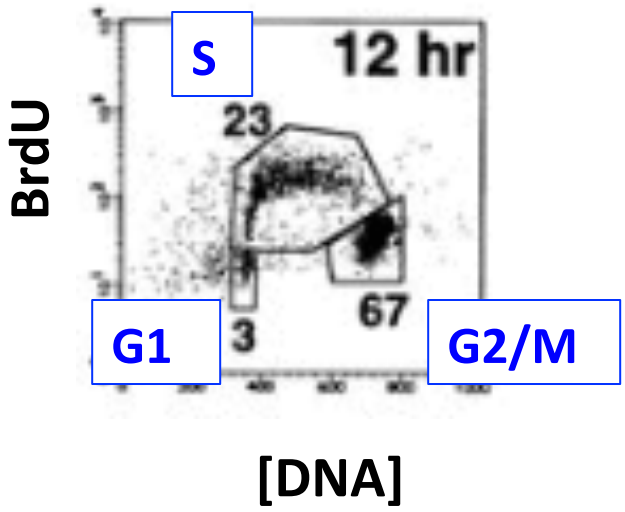


Image: E. Sonoda et al. (1998) *Embo J* **17**:598

FC to save a little money

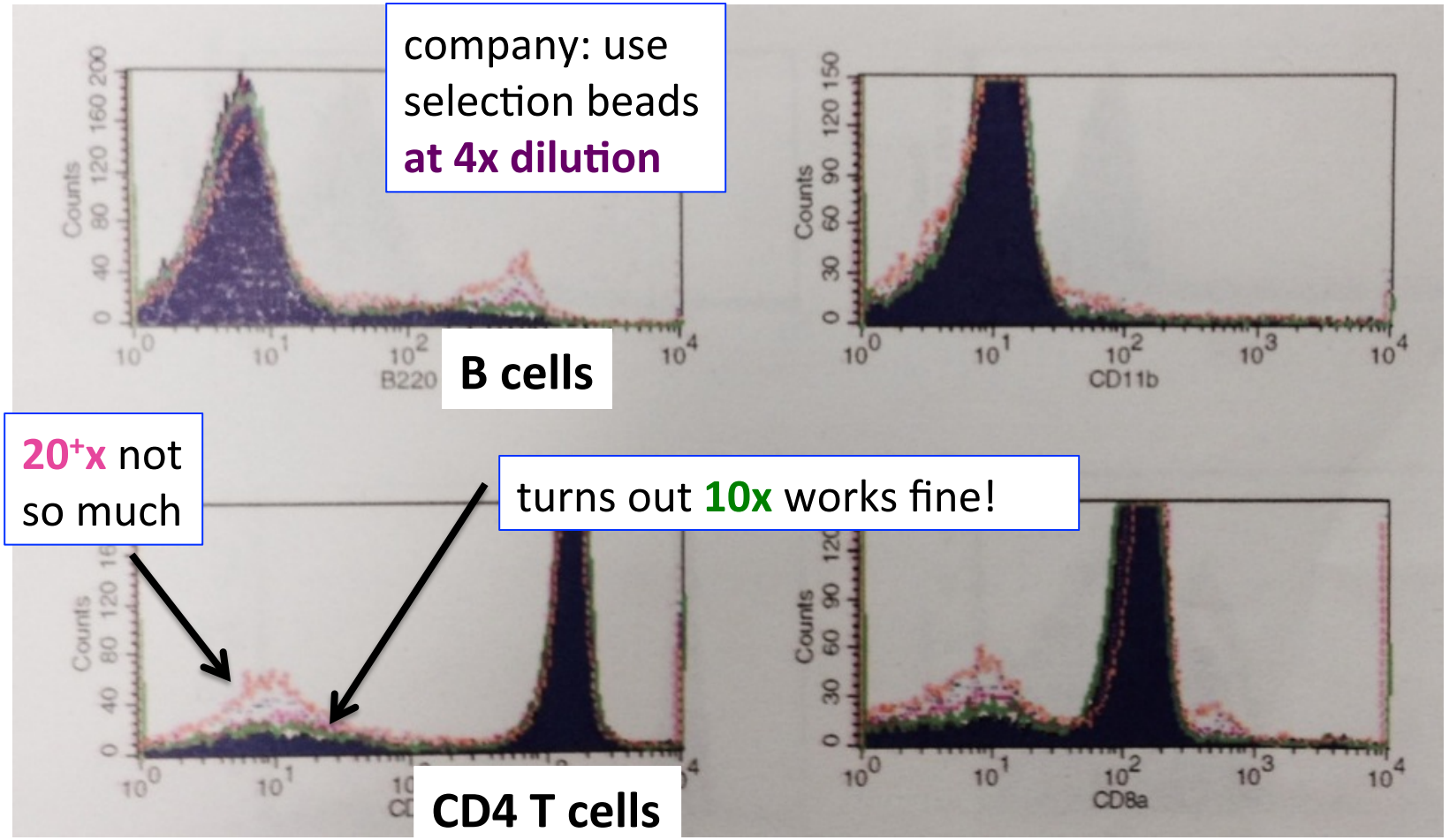
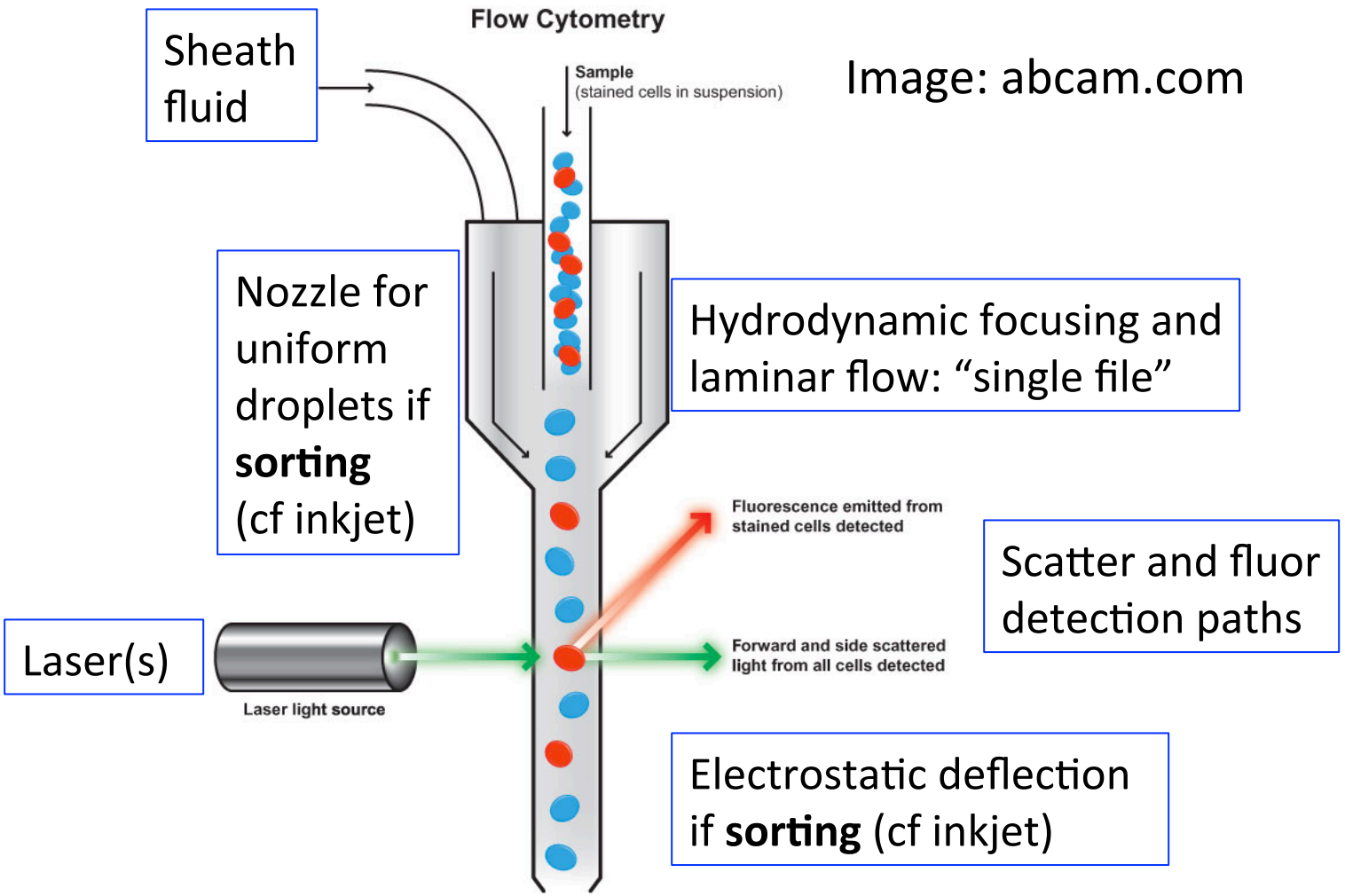


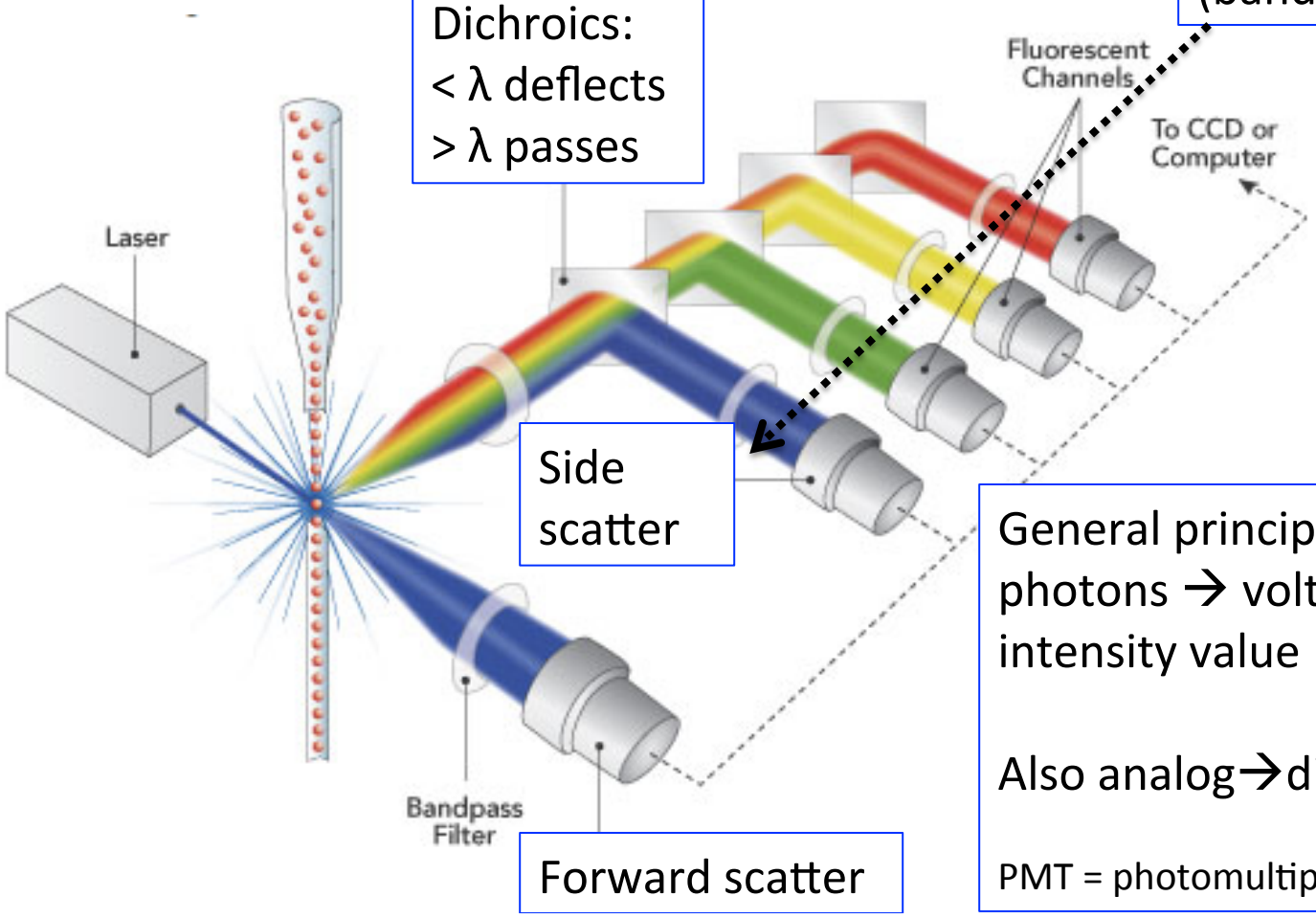
Image: A. Stachowiak

HOW FLOW CYTOMETRY WORKS

Overview of FC mechanics



FC detector paths



Addn'l filters (band pass)

Dichroics:
< λ deflects
> λ passes

Side scatter

Forward scatter

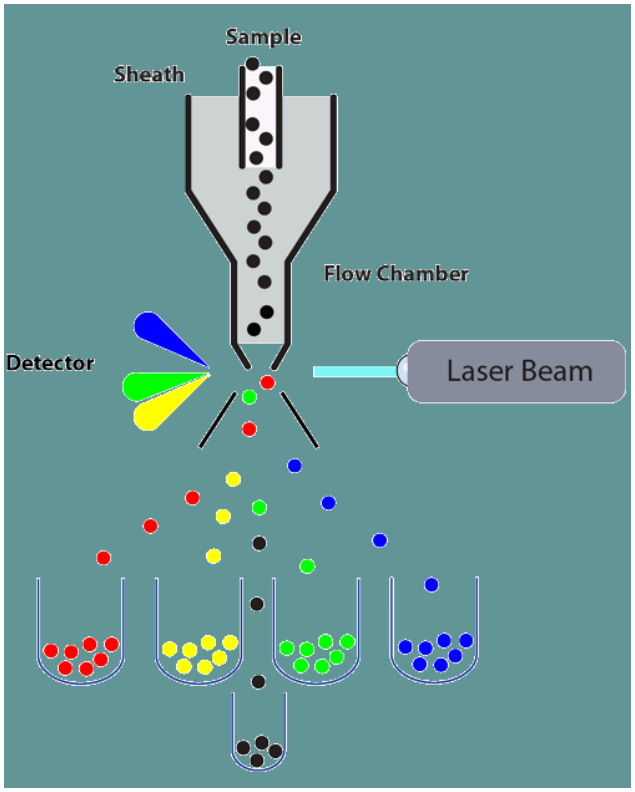
General principle: photons → voltage → intensity value

Also analog→digital

PMT = photomultiplier tube

Image: http://www.semrock.com/flow-cytometry.aspx

FACS operation visualized

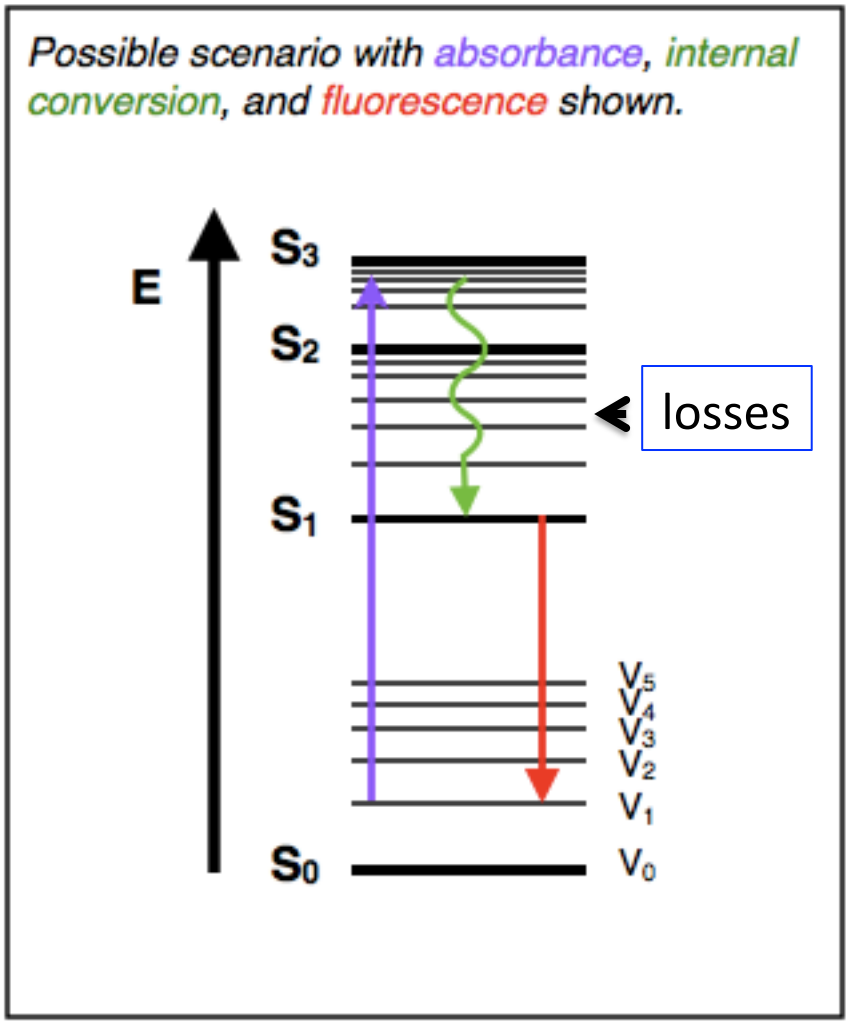


Fluorescence
Activated
Cell
Sorting

Some people call all flow cytometry FACS → don't let one of them be you! :)

Video: http://www.grc.nia.nih.gov/branches/lmg/fcl/new-index.htm

Brief review of fluorescence theory

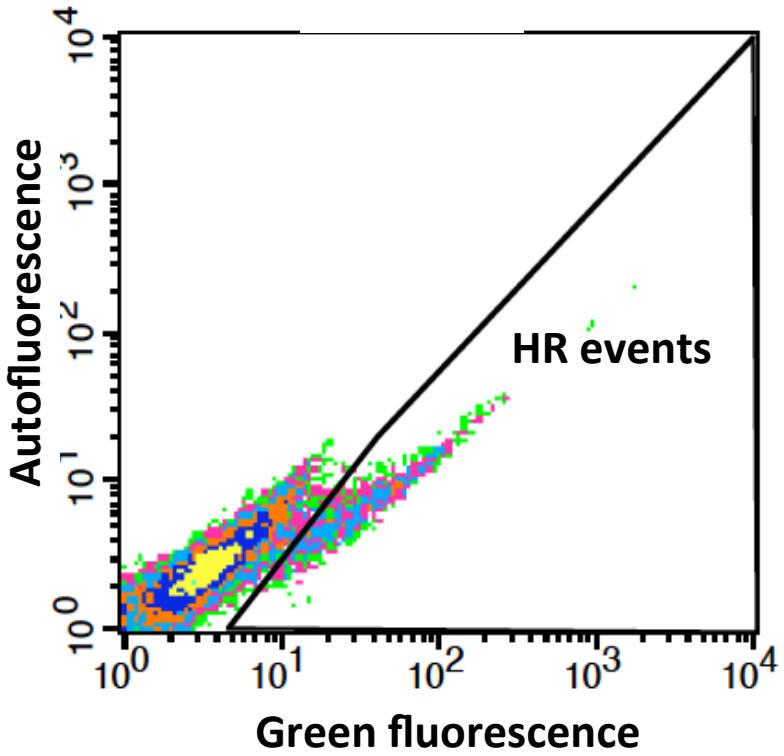


lower energy = higher λ

Image: http://chemwiki.ucdavis.edu/Physical_Chemistry/Spectroscopy/Electronic_Spectroscopy/Jablonski_diagram

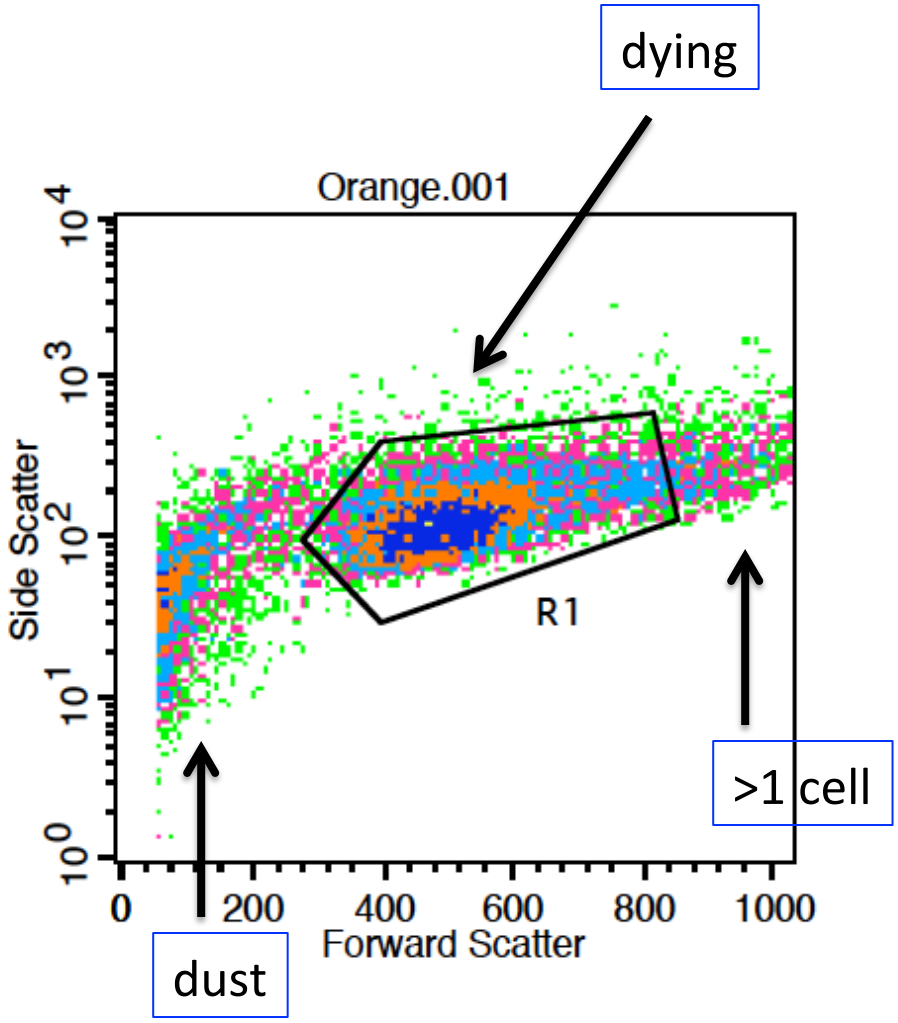
FLOW CYTOMETRY ANALYSIS

Lots of steps to get from here to there



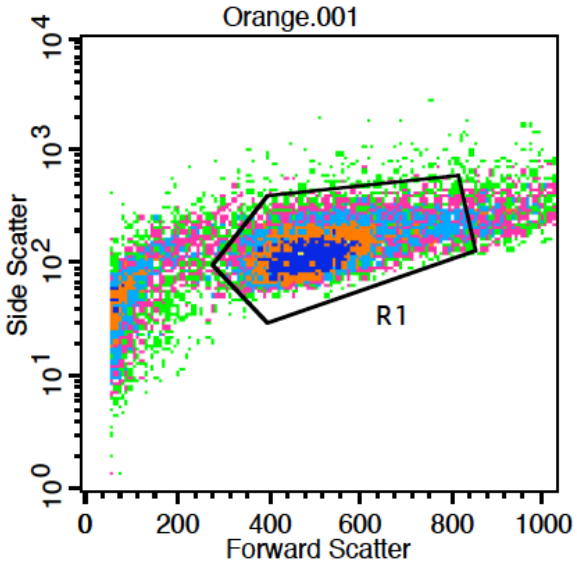
Setting scatter gate

- Forward scatter
  - size
- Side scatter
  - structure/complexity/ density/ granularity
- Overall selection goal
  - live cells
  - not dust
  - not dead cells
  - not aggregates

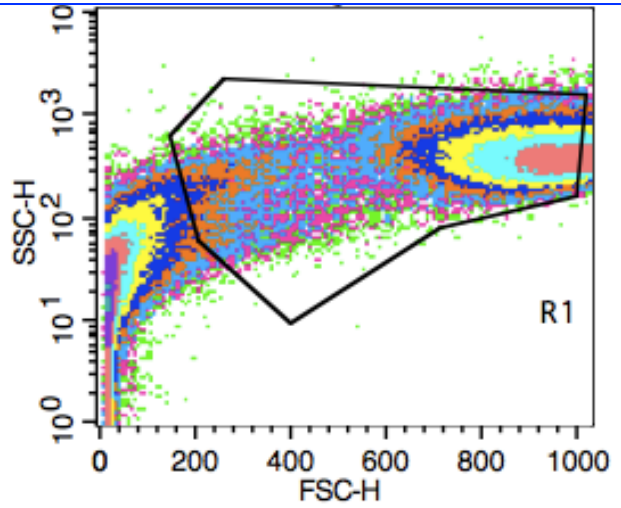


Run settings vs. analysis settings

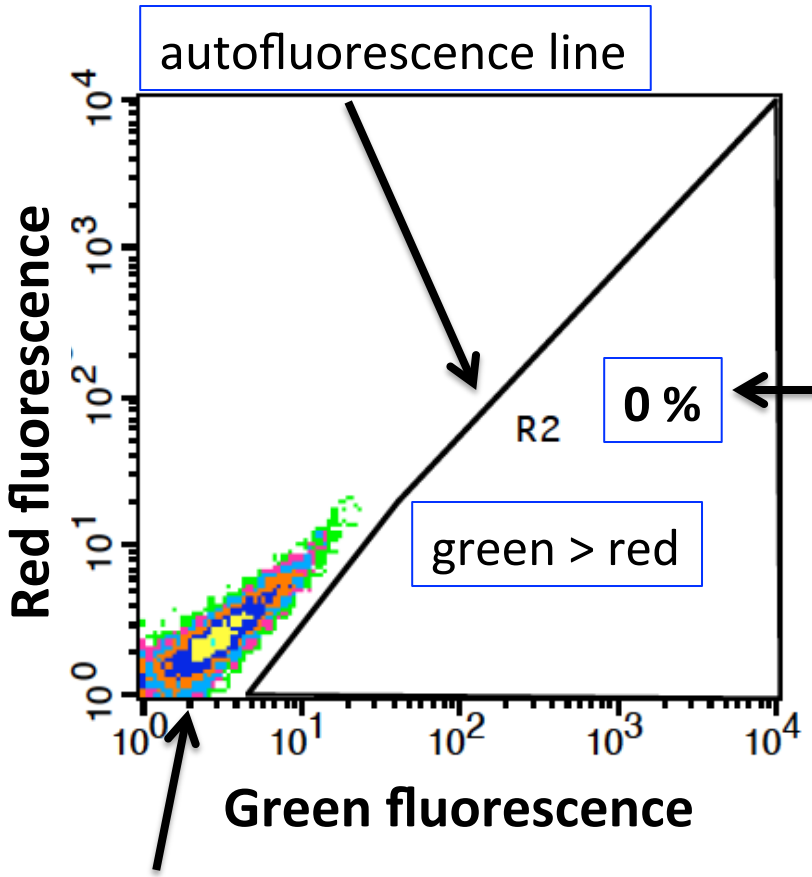
- During run: set PMT voltages to keep cells on scale
- Analysis settings – gates – can be fixed later
- Run settings can't!



What's wrong w/this picture?



“Mock” treatment sets negative gate

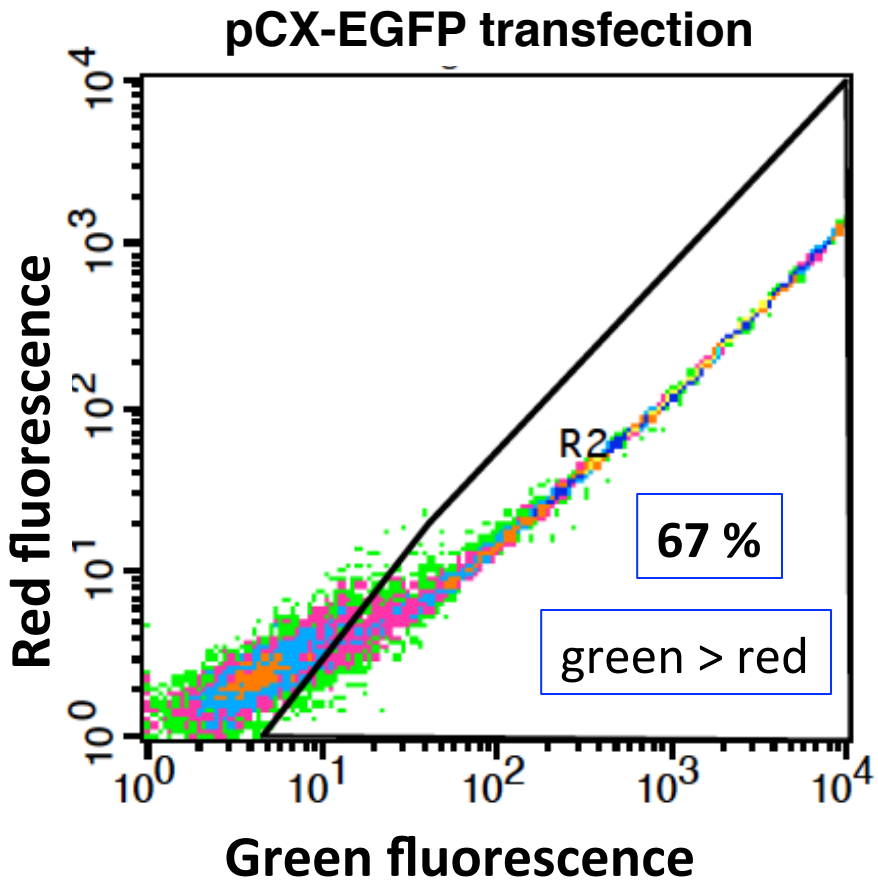


Importance of using the mock sample

- Treatment, such as lipofection, may alter:
  - scatter profile
  - autofluorescence
  - (including via cell death)
- Thus, mock is appropriate reference
  - NOT untreated cells
- Additional negative controls
  - confirm D5 or D3 **functional** deletion rather than *assume* it

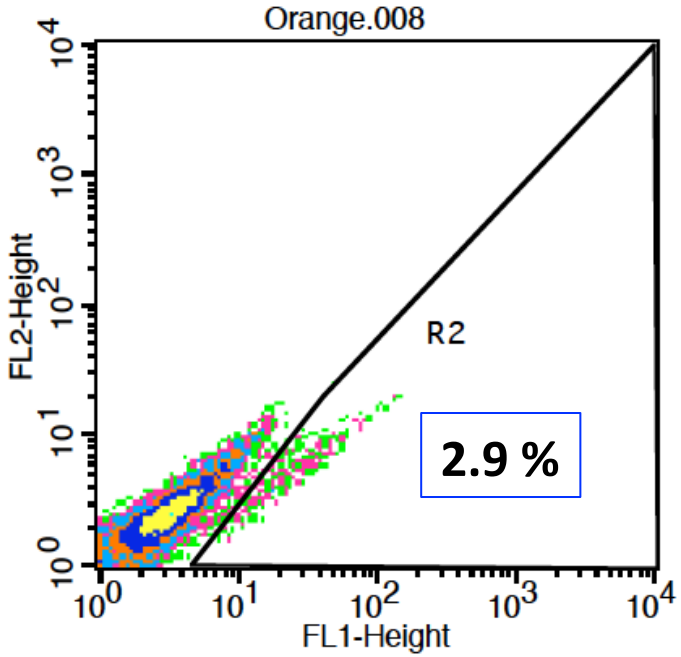
Single-color control confirms positive gate

- What if R2% of Team 1 > Team 2?
- **Control for transfection efficiency!**
- How can we use this information?

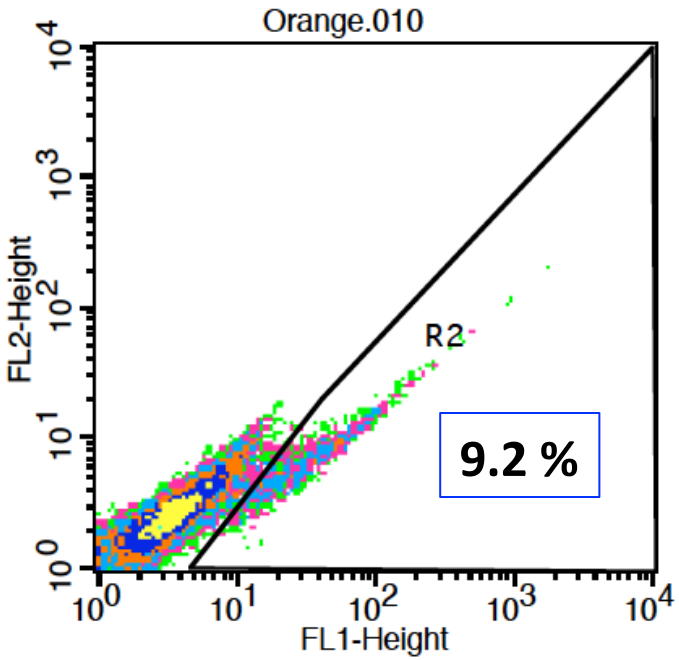


HR experiment sample data

Condition 1



Condition 2



More complex analyses: multi-color

- Koch facility has machines with
  - 4 lasers
  - **10-14** color capability
  - 96-well sample handler

BD Biosciences LSR II

- Yours will be a baby benchtop machine

- More commonly, folks use 2-4 colors
  - why is each color addition harder?
  - more controls and more analysis

Fluorescence compensation theory

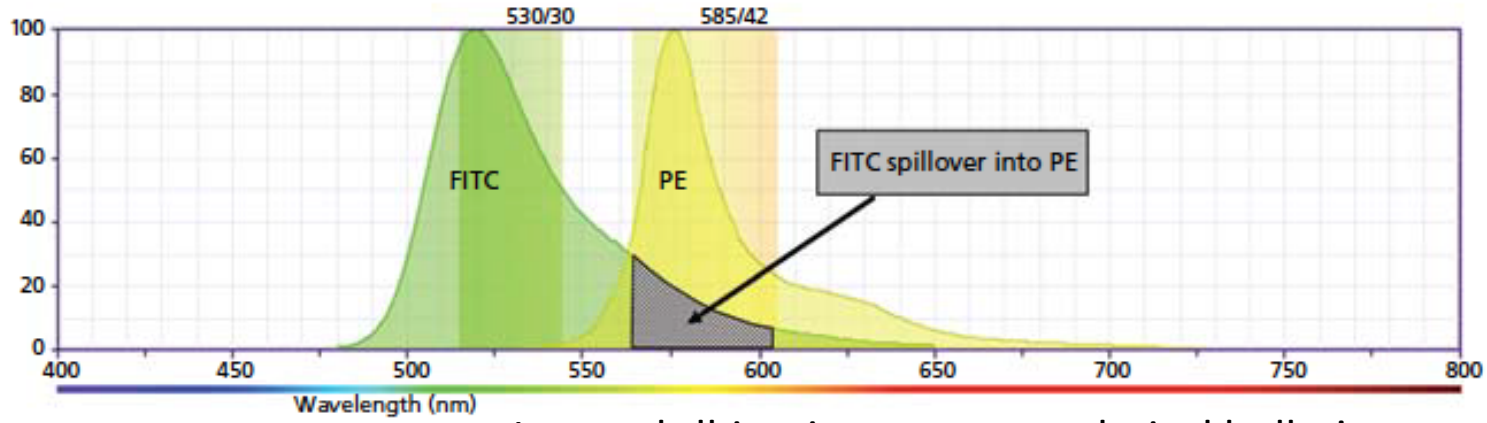


Image: bdbiosciences.com technical bulletin

Basic [intensity] equation:

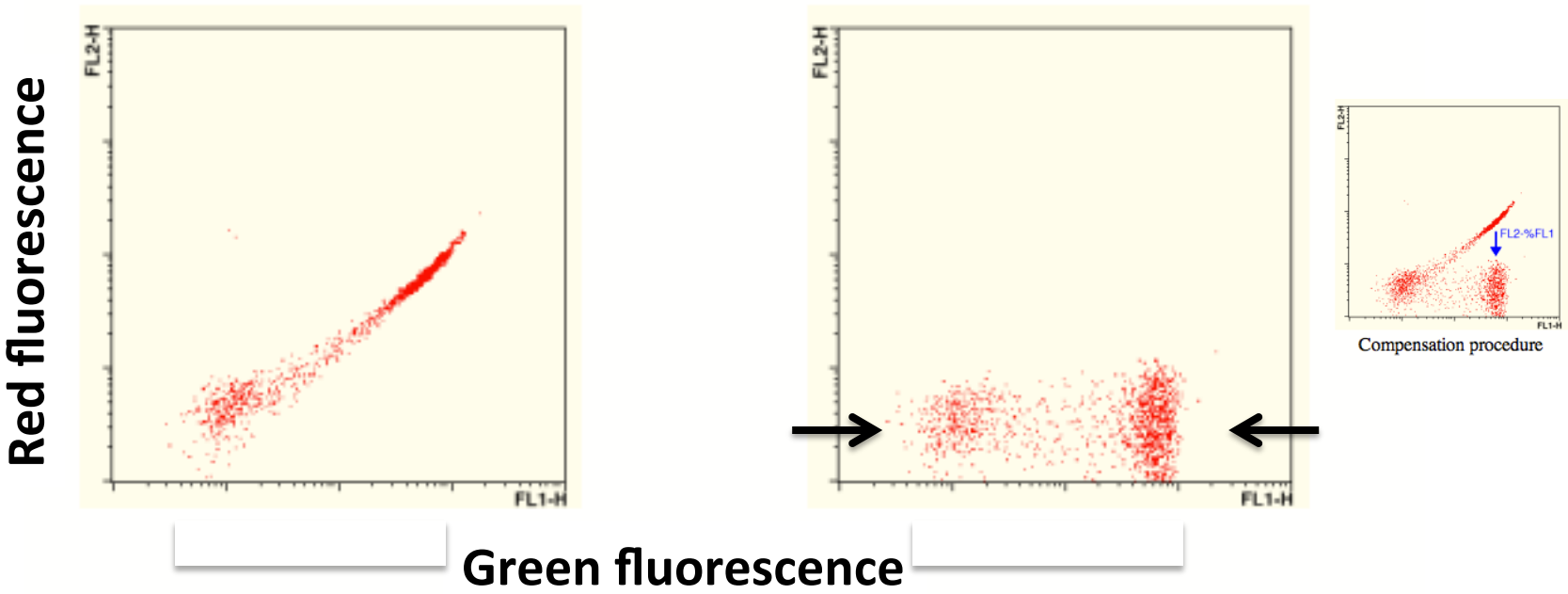
True [Red] = Measured [Red] – X % Measured [Green]

where X determined via single-color* controls

Determining compensation amount

Green “bleeding into” Red

Compensated!



Goal: equal MFI (median fluorescence intensity)

Images: http://flowcyt.salk.edu/howto/compensation/compensation-howto.html

It’s true though: “harder than it looks”

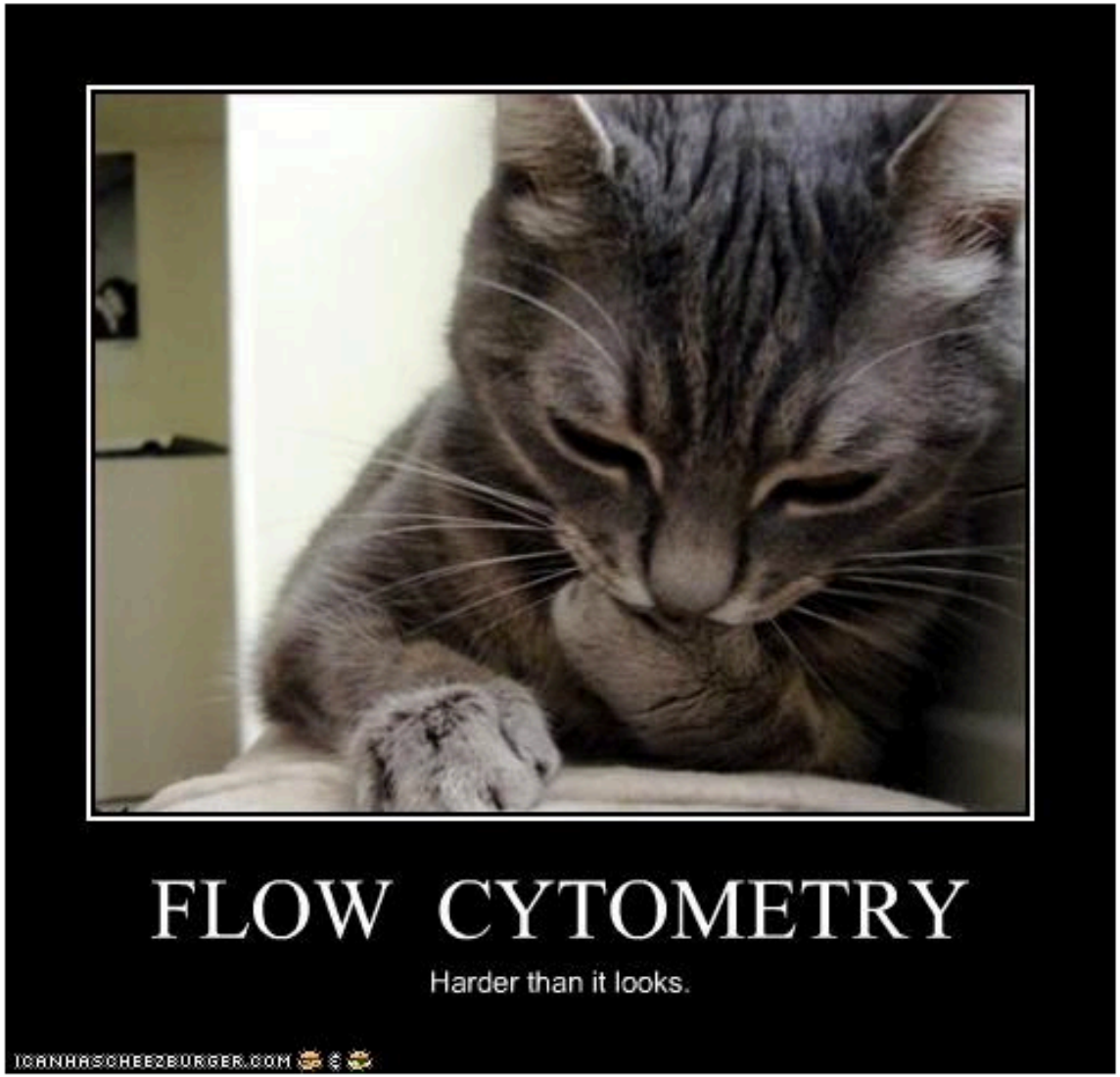
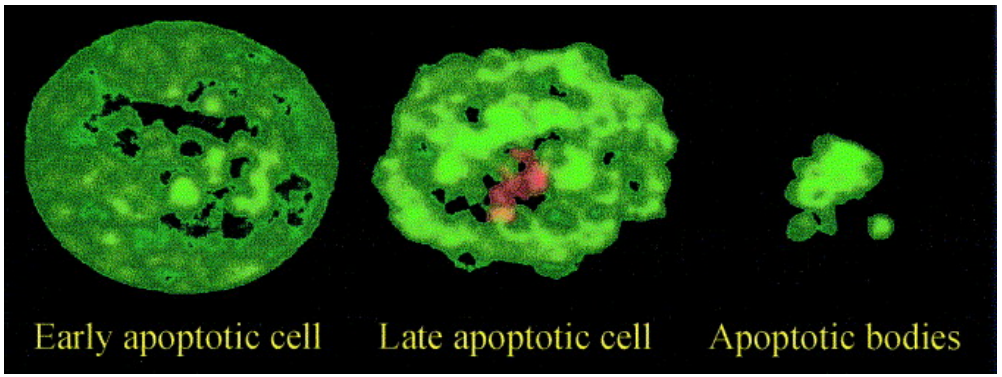


Image: catstar68

Flow cytometry versus microscopy

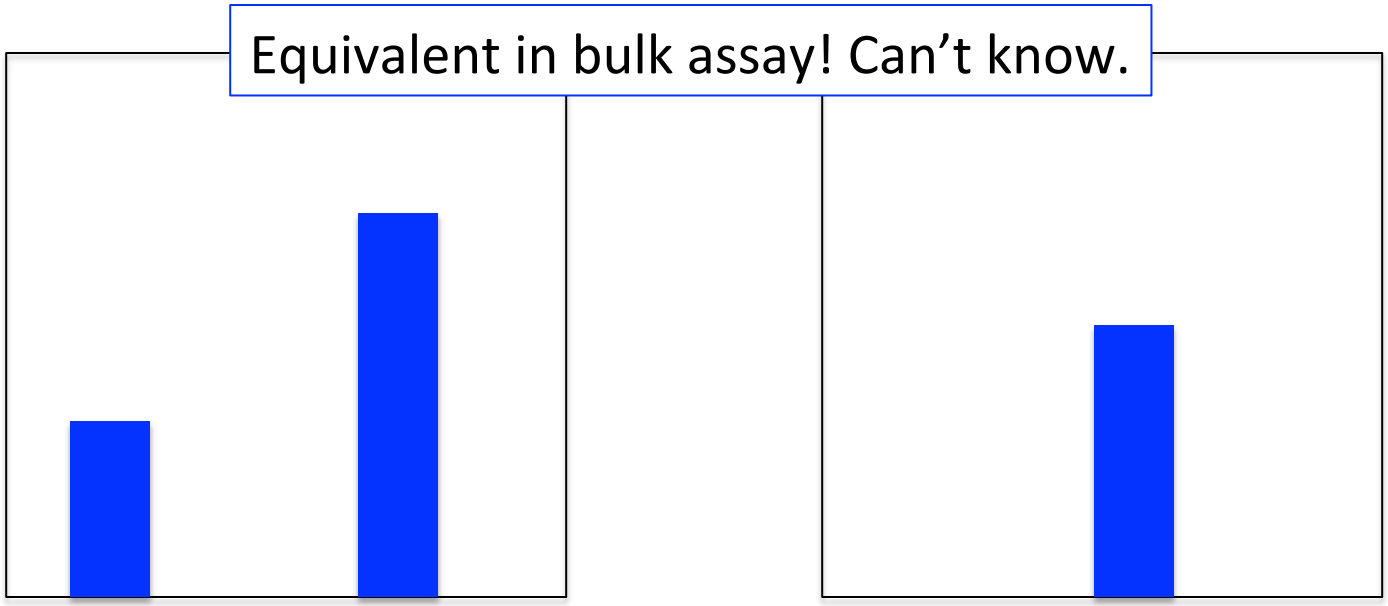
- Some FC pros
  - efficient data collection (scales well, >1000 events/sec)
  - efficient, credible data analysis
- Some µscopy pros
  - additional information (e.g., localization)
  - dynamic experiments have easier workflow



I Vermes et al. (2000)
J Immunol Methods
243: 167 Apoptosis

Single-cell vs. mean population assays

- *Both* FC and microscopy are single-cell assays
- Major pro compared to bulk/population assays: identify bimodal vs. peaked vs. other distributions



Hints for flow cytometry prep

- Aspiration technique
  - remove all liquid but don't linger
  - *clean* Pasteur pipette between conditions
    - ethanol dip, OR
    - exchange yellow pipette tip
- Label tubes with **correct number** + your color
- Pipet well to mix cells, disrupt aggregates
- Plan a workflow with your partner <u>in advance</u>

Module 1 in review: techniques

- Primer design and PCR
- DNA purification from mixture
- DNA ligation and cloning
- Bacterial transformation
- DNA isolation from cells
- Analytical restriction digest
- Mammalian culture and transfection
- Flow cytometry

SO WHAT?

Module 1 in review: lab principles

My version

- Controls now save time later
  - Ease interpretation
  - Focus troubleshooting
- Understanding protocol > black box
  - Ease interpretation
  - Focus troubleshooting
- Take a systematic and holistic view

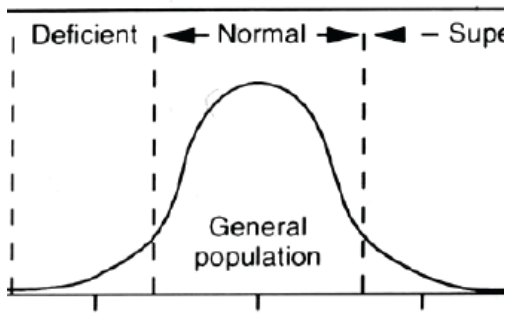
Module 1 in review: lab principles

Bevin's more fun version

- Nothing is 100%
- Ask "what else might be happening?"
- Avoid assumptions (controls!)
- Double-check at every opportunity
- Ask the same question in several ways

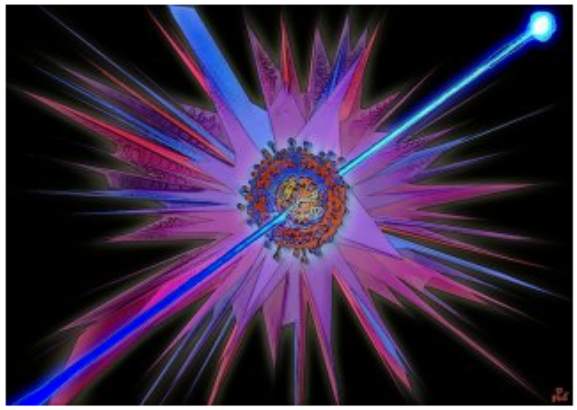
Module 1 in review: concepts

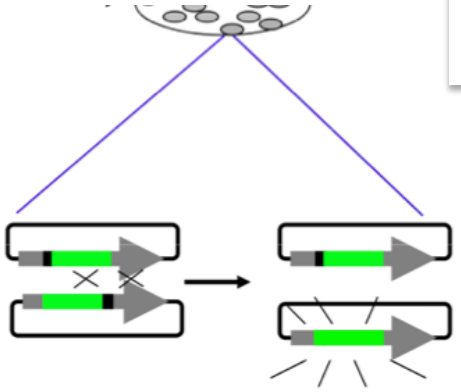
- Ubiquity of DNA damage
- Variety of repair responses
- Individual variation in
  - Exposures to damage
  - Strength of each pathway
- Implications for cancer
- Utility of an HR assay
  - If measure healthy cells exposed to UV?
  - If measure tumor cells exposed to chemotherapeutics?
  - Your idea here! → M1 'implications & future work' section



Module 1 Lecture 7: flow cytometry

- Flow cytometry is a biology and BE workhorse
  - cell identity, cell function, cell sorting
- FC operation is non-trivial
- FC analysis is non-trivial as # of colors ↑
- Your learned a lot in M1! Get excited to show it.
  - Or maybe missed some points. Don't hesitate to ask.





Next time:
Start Mod 2!