Caltech Flow Cytometry and Cell Sorting Facility

Who we are?

Michael Gregory	**–**	**Director**
Maddy Adolf	**–**	**Cytometry Technician**
Olivia Finney	**–**	**Cytometry Technician**
Shelley Diamond	**–**	**Emerita Director**
Ellen Rothenberg	**–**	**Faculty Supervisor**

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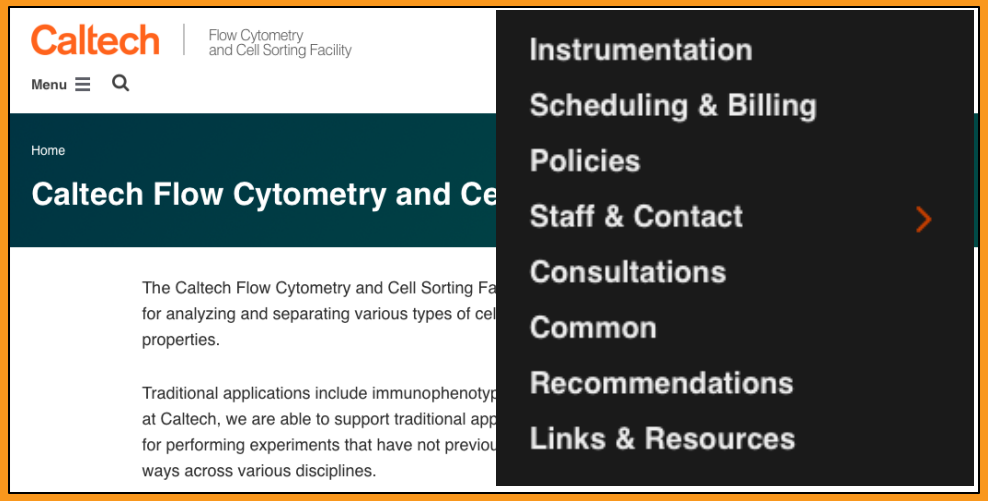
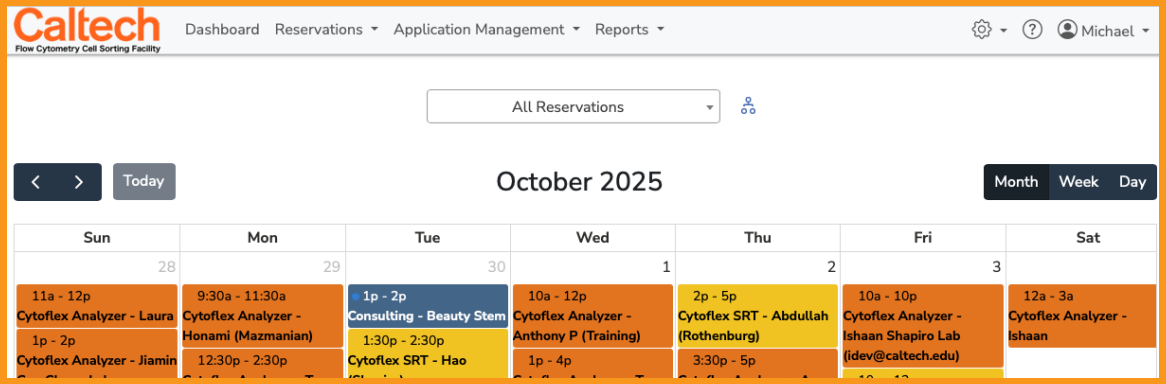


https://cellsort.caltech.edu

Getting Started

- New Project Consultation
  - Fill out our New Project form to schedule a consultation
  - Provide as much information as possible (we can update it during the meeting!)
  - Meet with the staff to discuss your project before you begin
    - BEFORE you order reagents, etc, as we may suggest changes!
- Sorts and trainings booked with staff (cellsort@caltech.edu!)
- Self-serve analysis booked directly on our scheduler



reserve.cellsort.caltech.edu

Flow Cytometry and Cell Sorting Facility Updates!

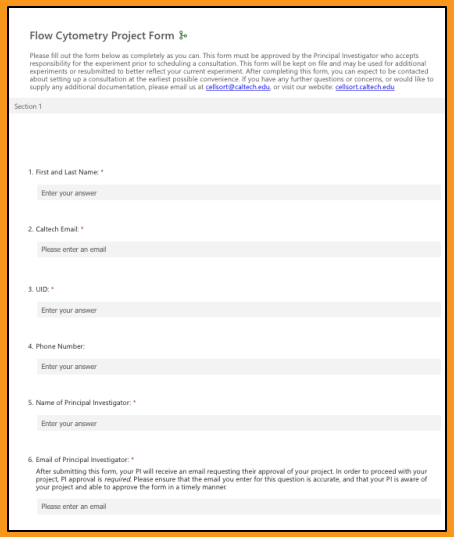
- New project form
- BioSorter large particle sorter
  - more on this later!
- Self-serve sort training
- FACSFusion downtime
- Retiring SY3200 and MACSQuant VYB
- Potential upgrades

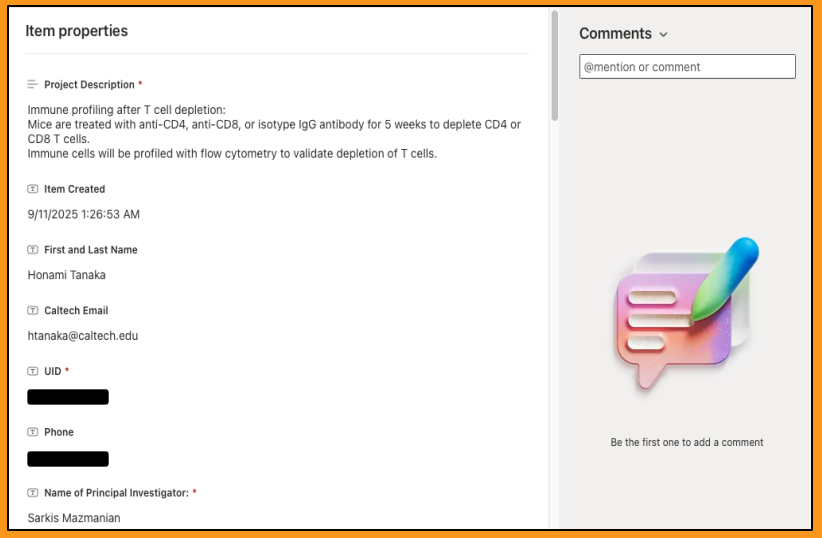
Updates!

New Project Consultation form

- Input via Microsoft forms
- Responses transferred to Sharepoint list
  - Modifiable with tracking – entries are confirmed and updated during consultation or when project changes
  - Collaborative – PI and other parties can be given access to view/comment
  - Keeps all staff members

    up-to-date on the project details
  - Searchable





Updates!

Self-serve sort training

- Advantages
  - More flexible scheduling – allows for afterhours and weekend sorting with appropriate training
  - Training customized to needs
  - Less expensive/hour
- Disadvantages
  - On your own for troubleshooting
  - Longer training period, with independent use only possible once objective benchmarks are satisfied

- Possible on FACSFusion and Cytoflex SRT (requires additional setup fee). BioSorter will be primarily self-serve!

Updates!

FACSFusion excessive downtime

- Previous service provider unable to repair instrument fully in ~2.5 months. Returning to manufacturer (BD) service contract.

Retiring SY3200 sorter and MACSQuant VYB analyzer

- VYB coverage ended earlier this year. Still available, but may not be able to repair if it breaks!
- SY3200 coverage will end in December. Still available, but most sorts will need to be moved to Fusion and SRT
  - Loss of capabilities –
    - UV laser
    - stream-in-air
  - Let us know if these capabilities will be require for your future projects!

Upgrades?

- New analyzer? Spectral instruments?
  - More colors (5 laser systems can get to ~40 colors!)
  - Full spectrum means autofluorescence can be better characterized
    - More possibility for label-free identification
  - Aurora by Cytek, ID7000 from Sony, Mosaic add-on for Cytoflex
- New Sorter? Stream-in-air? Spectral? Microfluidic? Imaging?

- Let us know your needs for upcoming projects to help us decide priorities!

- Labs with their own instrument that would rather not have to manage them? Happy to talk about mutually beneficial solutions!

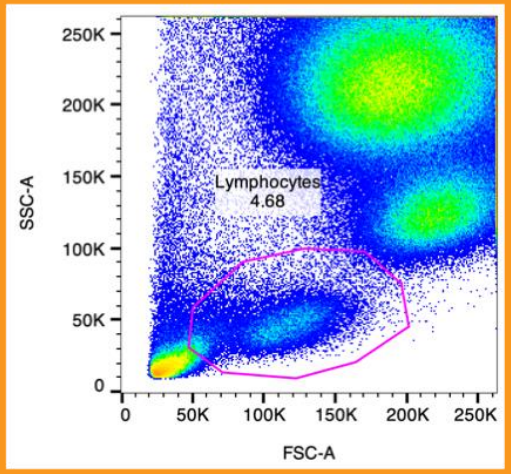
- Design/development groups on campus? Please connect us!

Flow Cytometry Data

- Flow cytometry data is stored as a list mode file:

Id	Time	Plate	Row	Column	Source well		Clog	Sorted status		In Regions		TOF	Extinction		Green	Yellow
1	59	n/a	0	0	n/a	N	0	00001	308	76.9	0.33	0.16	0.22	9293	320	n/a
2	532	n/a	0	0	n/a	N	0	00001	395	115	0.38	0.12	0.19	11129	408	n/a
3	5156	n/a	0	0	n/a	N	0	00001	127	20.1	0.08	0.04	0.04	5184	136	n/a
4	30589	n/a	0	0	n/a	N	0	10001	3656	4593	341	33.9	16.7	50063	3672	n/a
5	30709	n/a	0	0	n/a	N	0	10001	4128	5052	207	21.9	22.4	48981	4144	n/a
6	31095	n/a	0	0	n/a	N	0	10011	3626	3889	178	33.7	411	43211	3640	n/a
7	31315	n/a	0	0	n/a	N	0	10001	4159	4877	729	75.2	31.6	47135	4168	n/a
8	31390	n/a	0	0	n/a	N	0	10101	3442	4445	158	16.4	13.2	49612	3456	n/a
9	31463	n/a	0	0	n/a	N	0	10011	3597	5072	228	52.7	761	59586	3608	n/a
10	31555	n/a	0	0	n/a	N	0	10001	3549	4684	274	27.6	19.8	53955	3560	n/a
11	31684	n/a	0	0	n/a	N	0	10001	3679	4242	350	33.0	19.2	47299	3688	n/a
12	31867	n/a	0	0	n/a	N	0	10001	3821	4105	211	20.3	15.8	47023	3832	n/a
13	31870	n/a	0	0	n/a	N	0	10001	3727	5068	345	36.8	23.5	58797	3736	n/a
14	31904	n/a	0	0	n/a	N	0	10001	3731	4332	405	47.3	21.2	48395	3744	n/a
15	32033	n/a	0	0	n/a	N	0	10001	4882	6558	613	67.8	25.2	53294	4896	n/a

  - Files can get very large, depending on the number of events AND the number of parameters recorded for each event.
  - Specialized software allows you to explore the data visually (also possible for RNA-seq data!)



Flow Cytometry Analysis Software

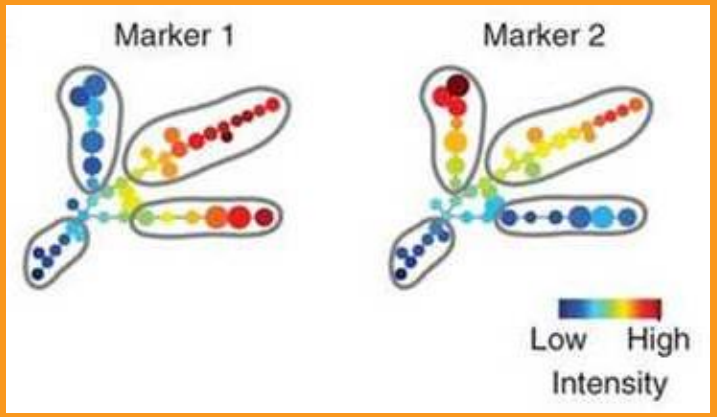
- FlowJo – Caltech site license (currently $310/yr)
- FCSExpress & OMIQ – Dotmatics
- Modfit & Gemstone – VSH - specialty software
- Kaluza & Cytobank – Beckman Coulter
- CytoScribe – BioLegend – brand new!

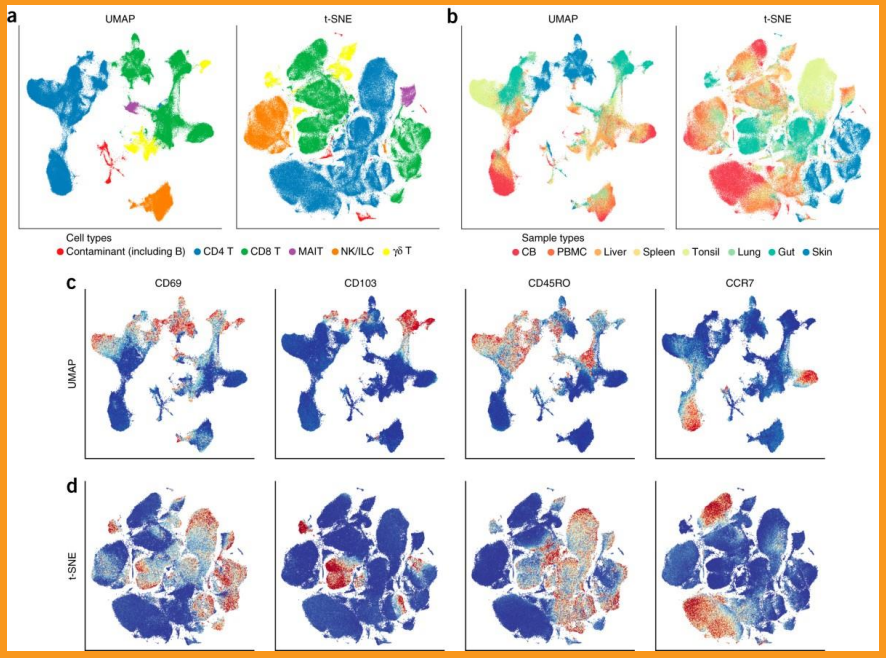
FREE!

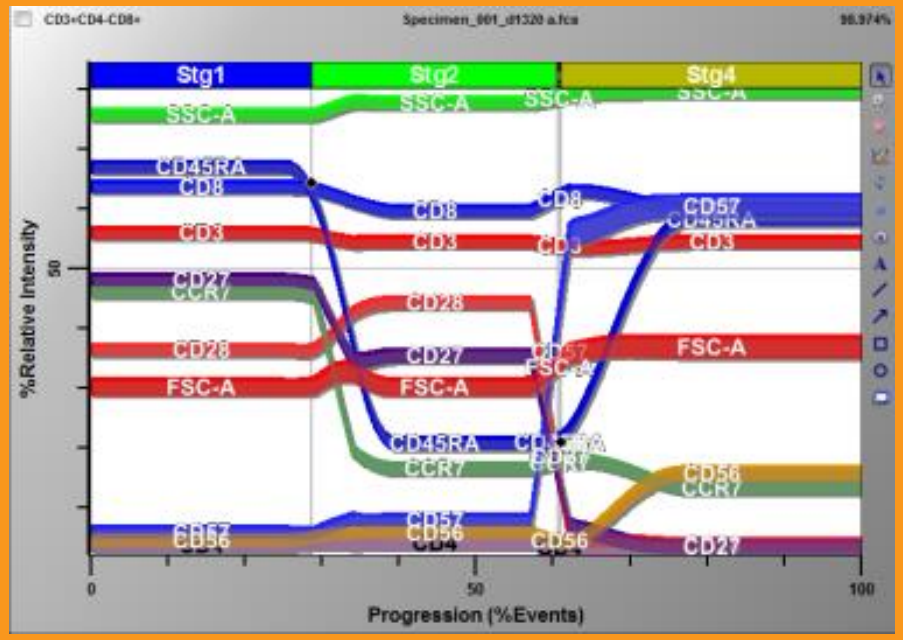
- Floreada.io – browser-based simple analysis tool
- R and Python analysis modules – these have gotten MUCH better
- Vendor acquisition software (ex CytExpert and FlowPilot)
  - Not recommended for complex analysis! Available on our Sharepoint!

Advanced Data Analysis Tools

- Useful for exploring high dimensional datasets
  - UMAP, tSNE, and viSNE
  - Gemstone
  - Spade

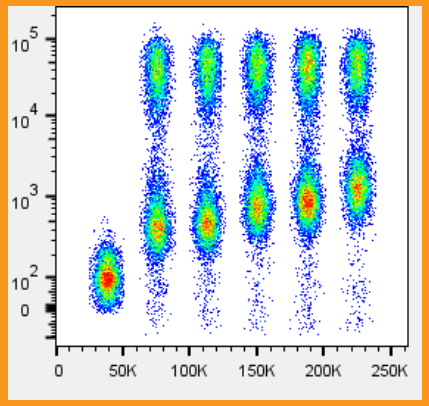


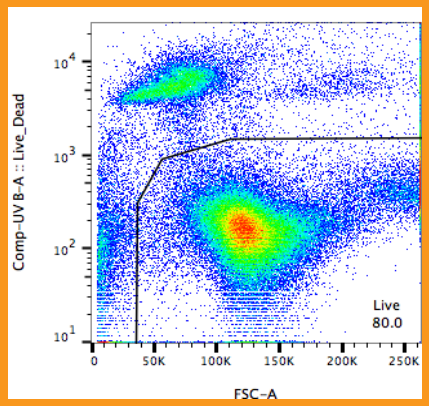


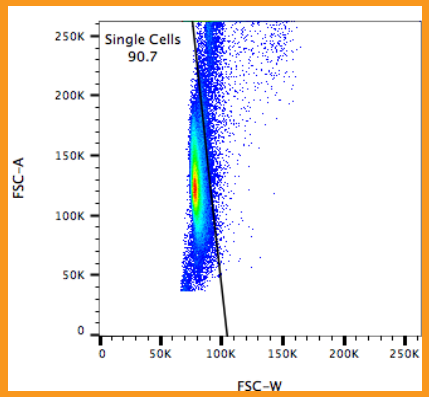


Preparing Properly

- Experimental Design
  - Titrating ab/dye concentrations
  - Including a viability dye
  - Debris reduction/removal
  - Proper controls
- Instrument Setup
  - Choosing Log/Linear for FSC/SSC
  - Setting threshold
  - Optimizing detector settings
  - Screen for doublets





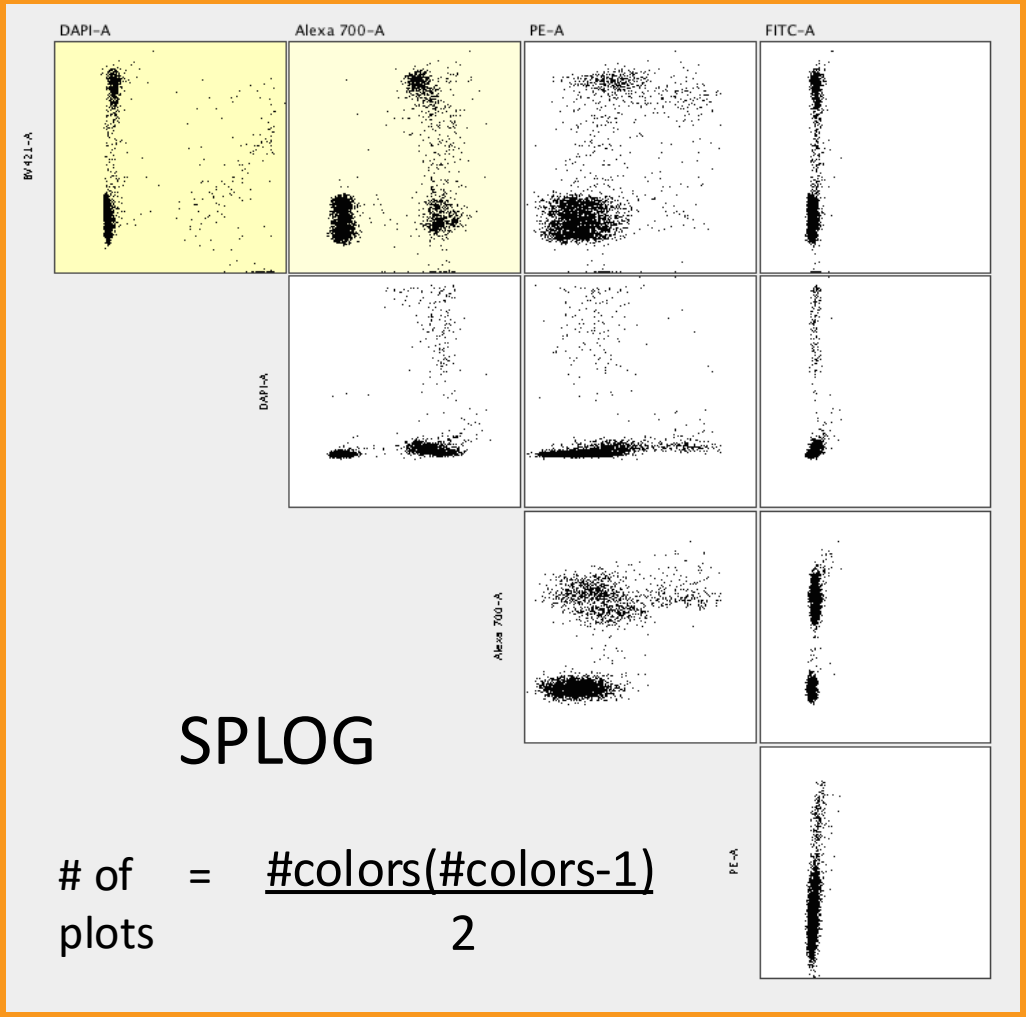


Voltages/Gains and Compensation

Setting Voltages

- Hardware on instruments have ideal voltages
  - Starting point, may not be ideal for your cells
- Find the best signal to noise
  - Voltration?
- Set voltages with compensation in mind!

NXN view



SPLOG

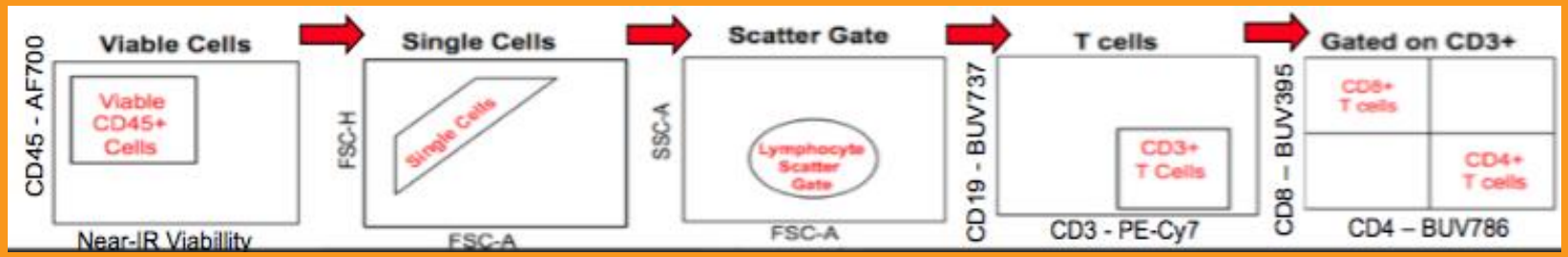
$$\#\text{ of plots} = \frac{\#\text{colors}(\#\text{colors}-1)}{2}$$

Proper Controls Ensure Good Setup and Gating

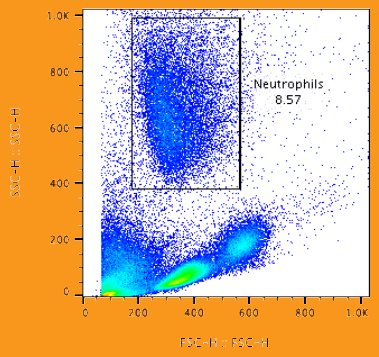
- Negative control – Cells without any staining
  - Used to set the baseline voltages for all colors in an experiment based on the natural autofluorescence of your cells
- Isotype Controls – Isotype-matched irrelevant antibodies
  - used to assess 'non-specific' binding of the non-antigen targeting regions of mAbs. Should NOT be used to set gating. Can inform on sample preparation issues (ie.blocking)
- Compensation Controls
  - Controls each stained with a single color, minus all others. Used to compensate for spectral overlap between fluorochromes
- FMO Controls – Fluorescence Minus One
  - Controls each stained with all colors, *except* one. Used to account for interactions between fluorochromes. A powerful & recommended gating tool for multicolor experiments

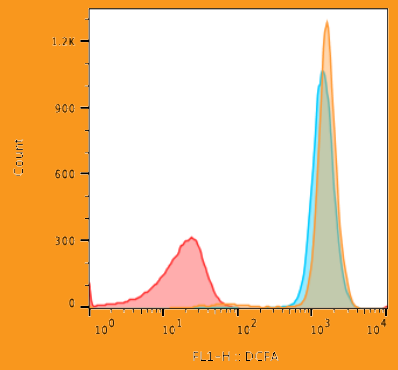
Data Analysis – How to begin?

- What's the question you're looking to answer with your data?
  - Phenotyping Subpopulations



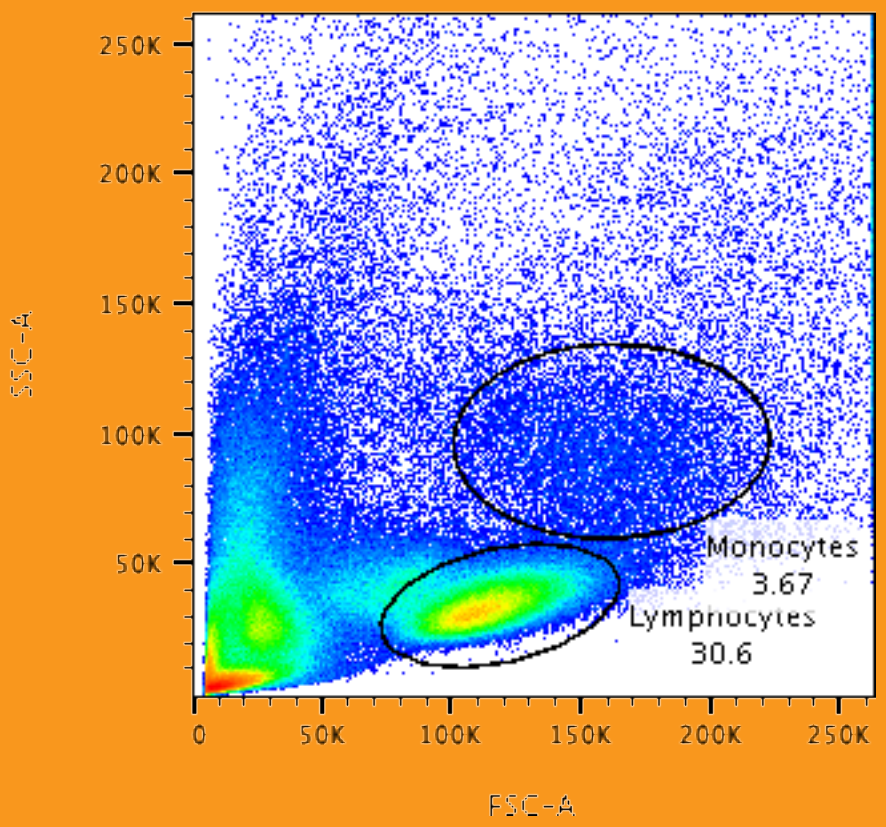
  - Mean (or Median) Fluorescent Intensity (MFI)
  - MFIR

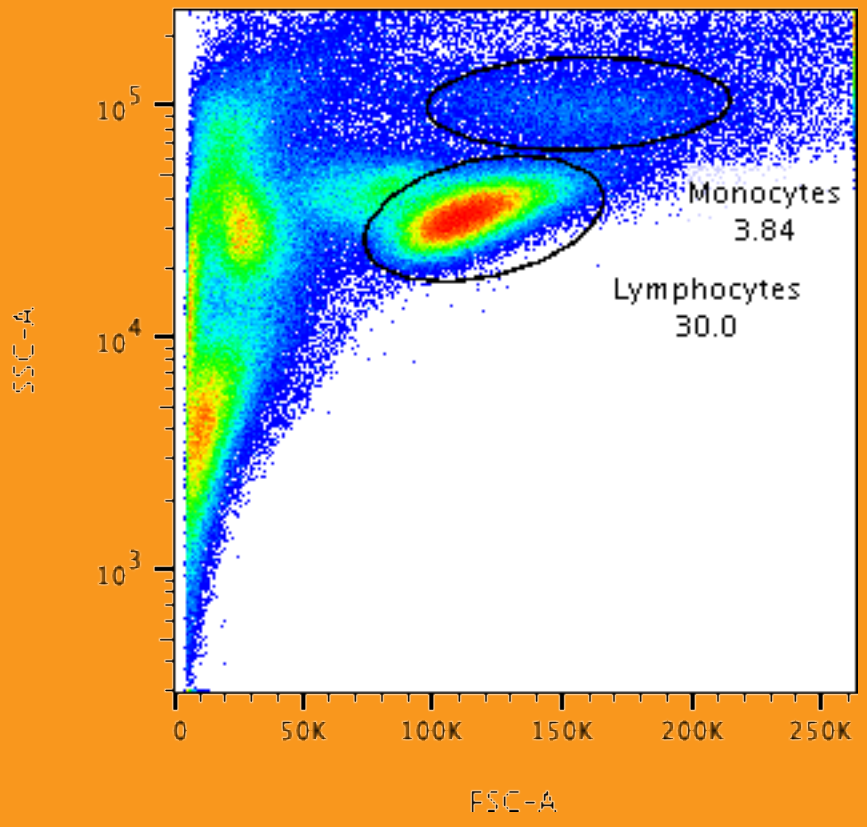




Sample Name	Subset Name	Count
ROS07:14.019 C3	Neutrophils	25321
ROS07:14.018 C2	Neutrophils	24801
ROS07:14.017 C1	Neutrophils	14909

SSC - log versus linear

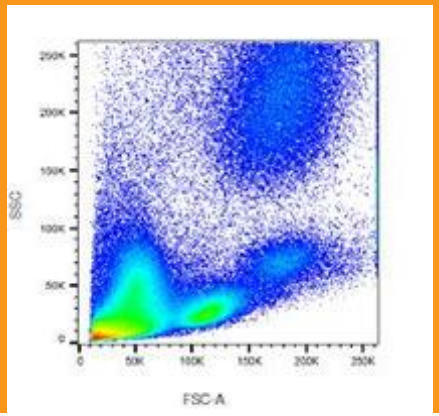


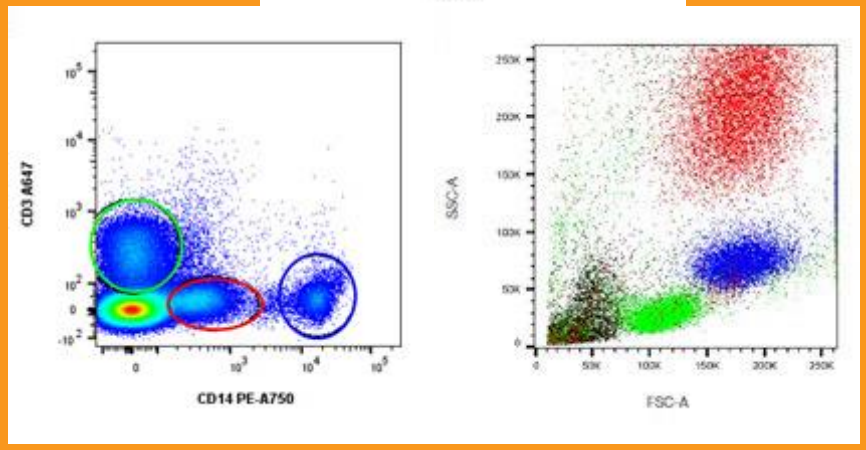


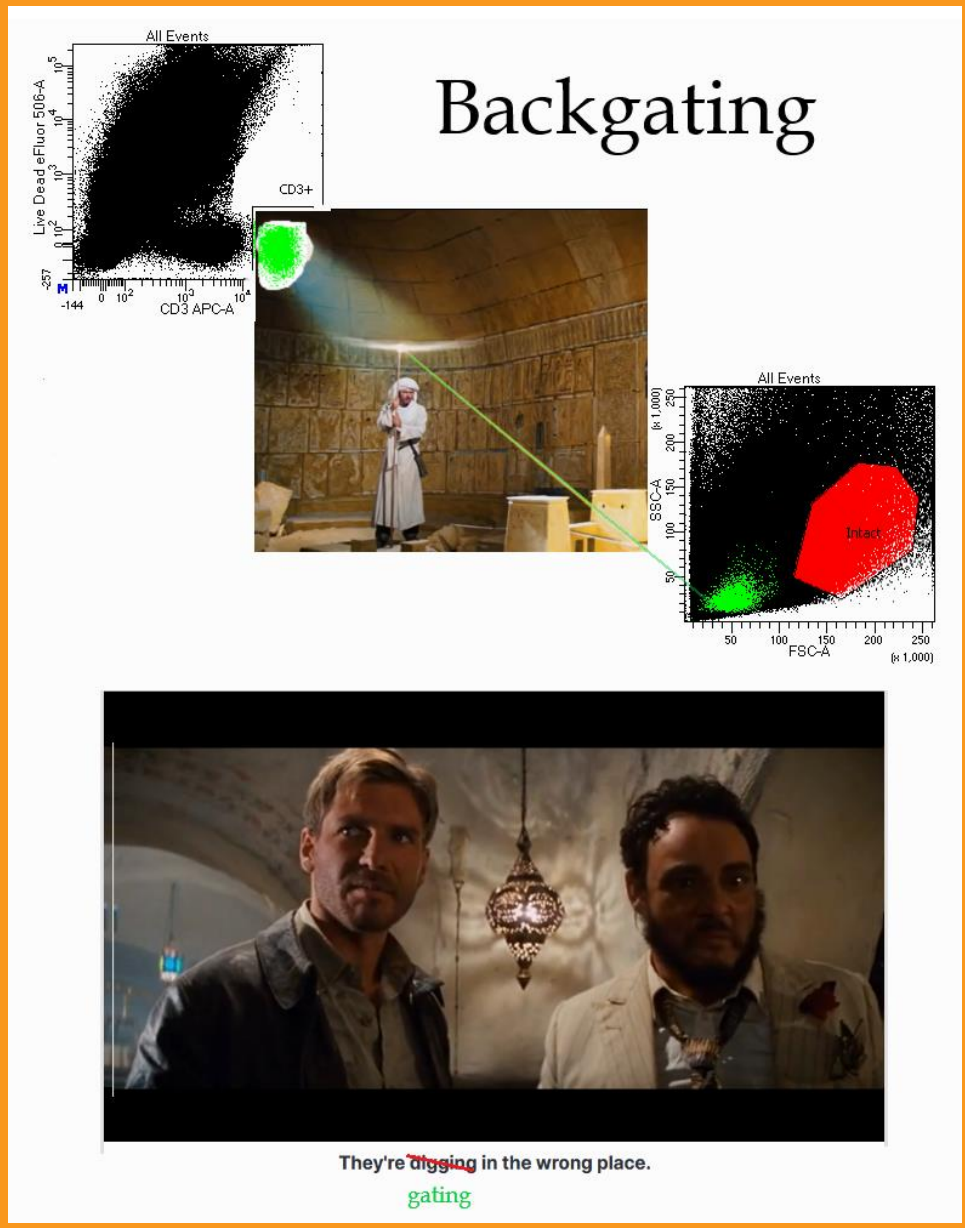
Newer instruments/software allow you to change the scaling after the fact, but if settings are optimized for one or the other you could be stuck.

Finding your Cells of Interest

- FSC v SSC – backgating – during acquisition AND/OR analysis.



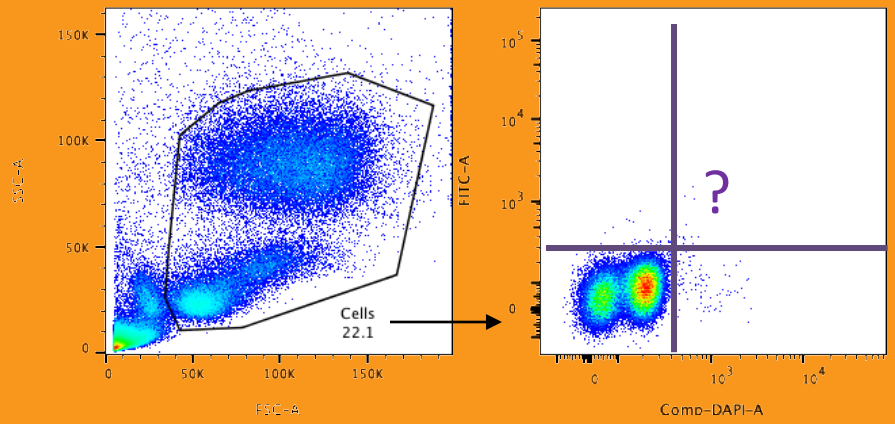


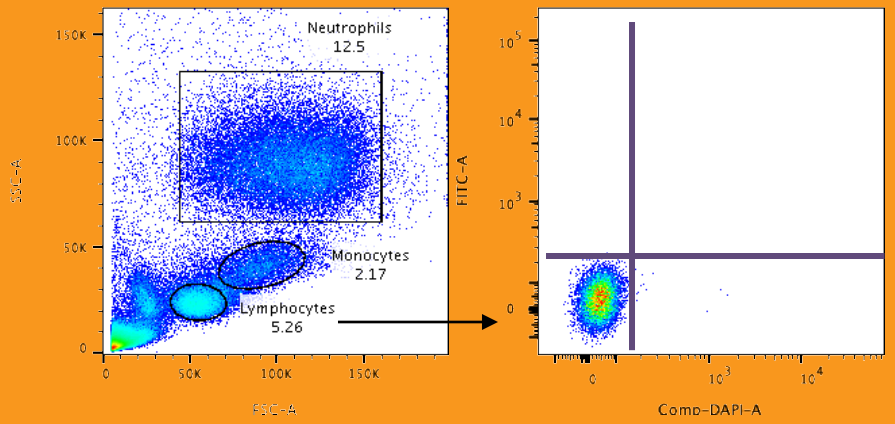


Data from Bio-Rad!

Finding your Cells of Interest

- Gating different scatter populations early?
  - Different cell populations may have different autofluorescence!

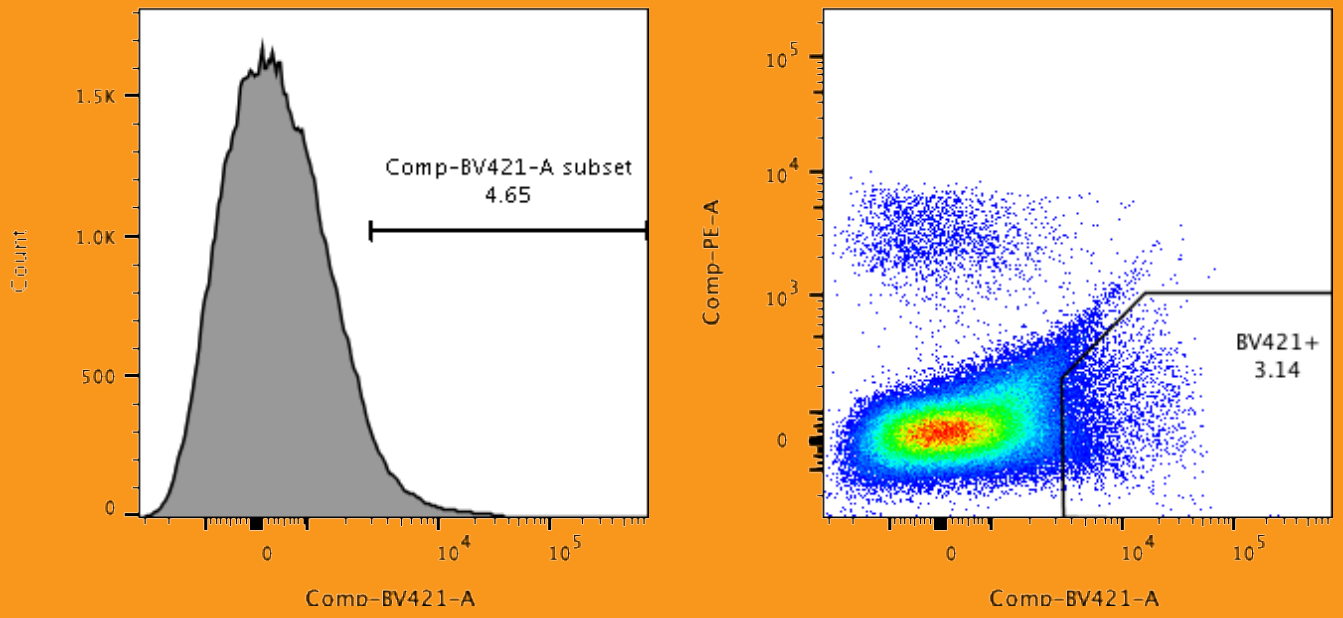




Thanks to Raquel Lopez Diez!

Finding your Cells of Interest

1 parameter vs 2 parameter plots help ID the real positives.



- Auto-fluorescent cells that were not removed by upstream gating can appear as a ‘spike’ that should be considered when gating.
- Spectral cytometers allow for autofluorescence subtraction or extraction.

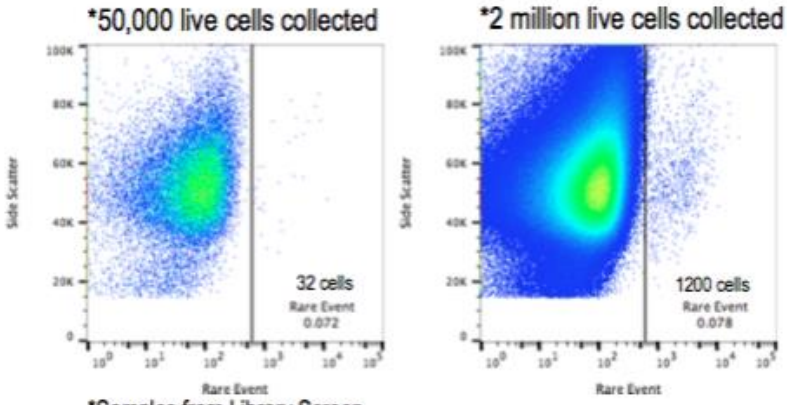
Number of cells to acquire?

- Must determine how many cells will be enough to analyze smaller subpopulations.

Precision, most commonly expressed in coefficient of variation (**CV**) values, is defined by Shapiro* (2003) as "The extent to which identical values are obtained from measurements of identical particles." (pg. 214)

Cell Count	Precision
10	+/- 31.6%
50	+/- 14.1%
100	+/- 10.0%
1000	+/- 3.2%
10000	+/- 1.0%

As the number of events collected increases, the variability in the precision of your data decreases.



*Samples from Library Screen

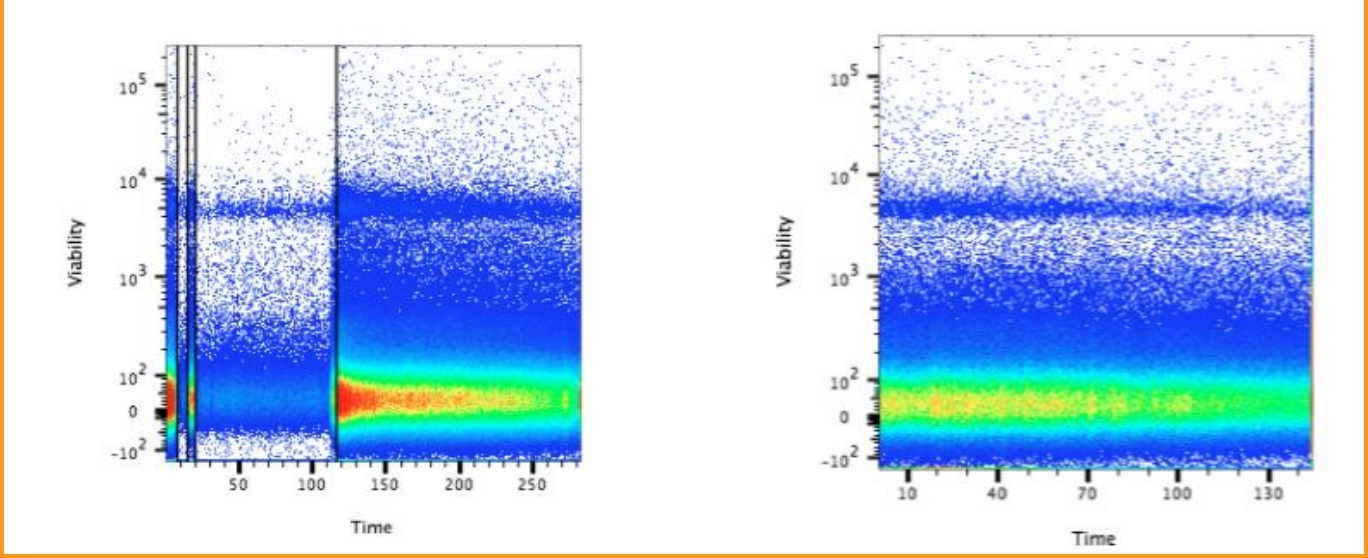
Precision of data is determined on your gated cells of interest.
i.e. If you have 500 total events, but out of that number only 10% are your cells of interest (50 cells), your precision decreases from +/- 4.4% to +/- 14.1%.
Note: An equal number of your negative control should also be collected.

Standard is to collect at least 1,000 gated cells of interest for statistical significance.

Thanks to Kathy Daniels, Regeneron!

Data QC!

- Time parameter - can use to assess and even salvage data!



flowClean: Automated Identification and Removal of Fluorescence Anomalies in Flow Cytometry Data

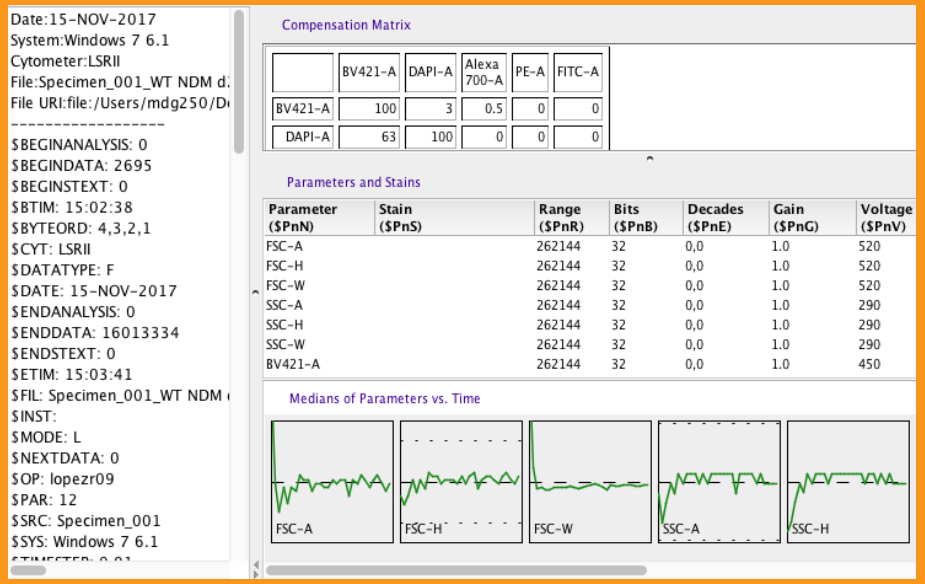
Kipper Fletez-Brant,[1,3] Josef Špidlen,[2] Ryan R. Brinkman,[2] Mario Roederer,[3] and Pratip K. Chattopadhyay[3,*]

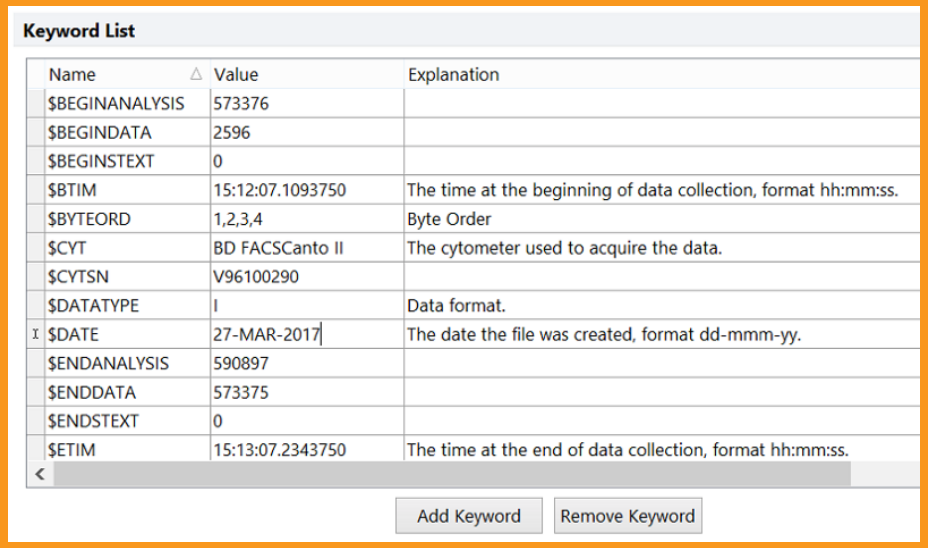
"Tracking cell populations in centered log ratio (CLR) space" to "flag time periods with fluorescence perturbations leading to the emergence of false populations."

Thanks to Kathy Daniels, Regeneron!

Automation for Longterm Projects

- Using Keywords
  - Who's seen these windows before?
  - Can enter custom keywords to help automate analysis
  - Easily map gates to data files based on their keywords.





MIFlowCyt

Preparing a Minimum Information about a Flow Cytometry Experiment (MIFlowCyt) Compliant Manuscript Using the International Society for Advancement of Cytometry (ISAC) FCS File Repository (FlowRepository.org)

Josef Spidlen, Karin Breuer, Ryan Brinkman

First published: 01 July 2012 | https://doi.org/10.1002/0471142956.cy1018s61 | Citations: 24

Experiment Overview	Purpose/Goal/Hypothesis
	Experiment Variables
	Conclusions
	Quality Control
Flow Sample (Specimen)	Material
	Source/Organism/Location
	Treatment
	Reagent/Analyte/Detector/Reporter
Data Analysis	List-mode Data
	Compensation
	Gating
	Descriptive statistics
Instrument Details	Instrument Identification
	Fluidics Configuration
	Optical Configuration
	Electronic Configuration

- **M**inimum **I**nformation about a **Flow Cyt**ometry Experiment
  - Published in 2012, following CYTO workshop
  - Annotates data with lots of essential information
  - Aimed to make (re-)use of public flow cytometry data sets more possible
- "Required" for FlowRepository - http://flowrepository.org
  - And some specific journals

Methods Section

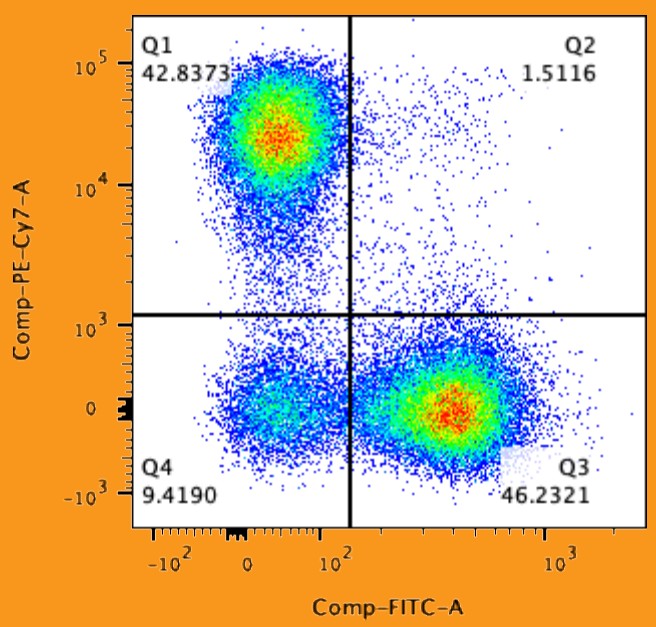
- Sample Preparation and Exp Design:
  - Sample source and preparation
  - Treatments
  - Labeling (ab clones, conc, incubation times/conditions)
  - Controls used,
- Instrument Setup:
  - Describe instrument
    - Manufacturer, model, software
    - Optical configuration
    - Fluidics setup (pressure, nozzle size, buffers used, etc)
  - Sorting conditions

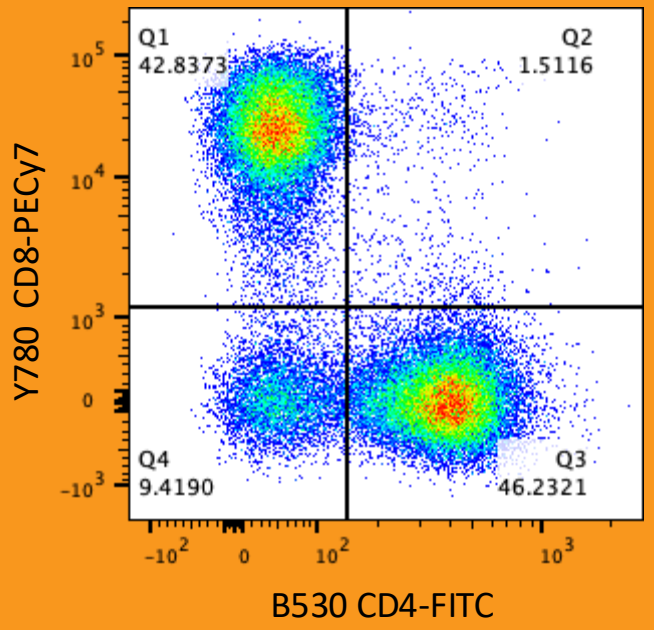
Methods Section

- Data Acquisition/Analysis:
  - How compensation or unmixing was performed
    - Antibodies, cells versus beads
    - Justification of changes/modifications
  - Number of events recorded
    - Smaller populations might require more events
  - Gating scheme –
    - Gating decisions - what controls were used to set these
    - Justification for adjustments, etc
  - Statistics
    - Which populations are included?
    - MFI = mean? Median?
    - Fold increase over control vs standardizing to a bead, etc

Results Section

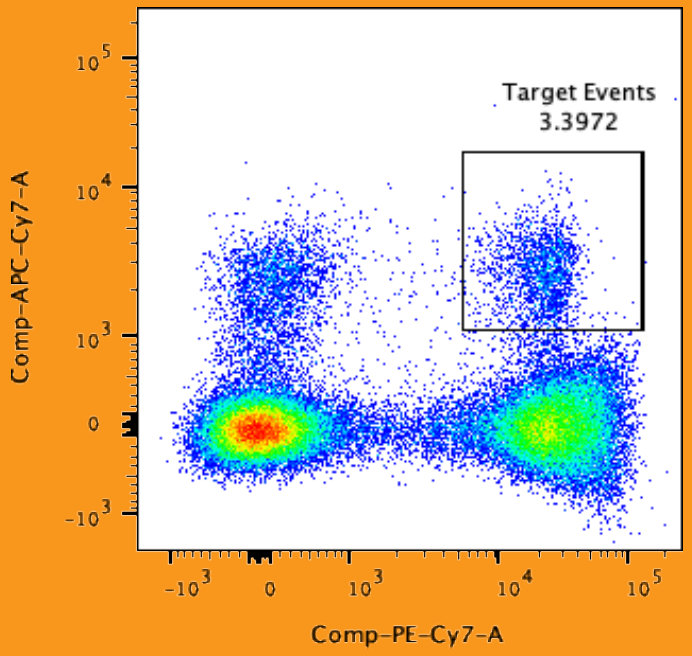
- Figures – Dot plots
  - Adjust axis labels to clearly convey what is being shown
    - Change generic names to appropriate label – usually the marker/antigen, fluorophore, detector name
      - Ex. CD45-FITC B530
    - Names can be changed using FCS editors or manually with image editors





Results Section

- Figures – Dot plots
  - Scaling of axis
    - Scaling should be consistent between samples
    - Same data, but visualized differently – confusing for readers



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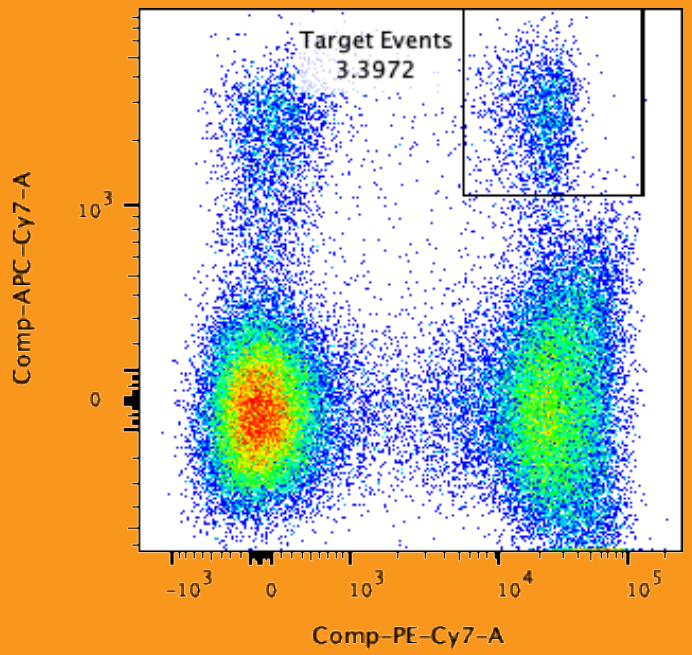
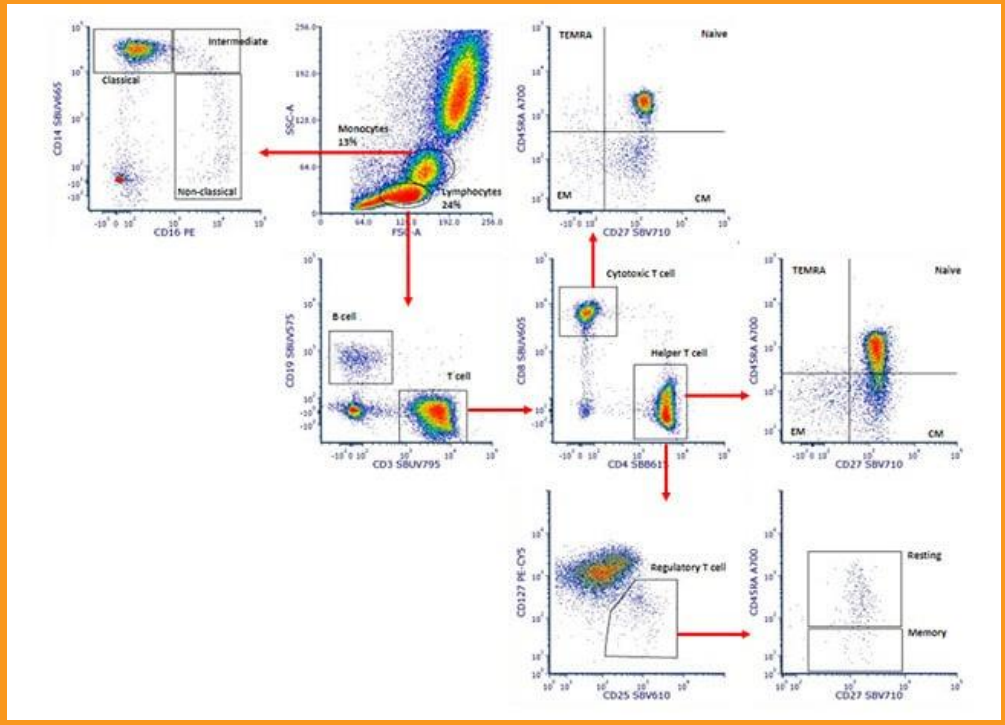


Results Section

- Gating Scheme
  - Full gating scheme should be included in supplemental data
  - FMO controls and other control gating may or may not be included, but raw data should be made available in public repositories like Flow Repository
- Alternative gating
  - Not always necessary

    to start with FSC/SSC
  - Start with Time, landmark

    gating (like CD45, DAPI, etc)



Publication Summary

- Aim to provide all information necessary to replicate experiment
  - MIFlowCyt guidelines are very comprehensive
- QC your data well and explore in a variety of analysis pathways
  - Backgate to make sure you are not missing populations
  - Be mindful of the upper right corner, cells can hide there!
  - Triple check your compensation before analyzing!
- Include gating scheme and gating justifications
  - Flow cytometry data can be presented as a generalized gating scheme, but deviations/adjustments need to be justified
- Plots
  - Label with Detector name, Marker/Antigen, and Fluorophore
  - Scale is arbitrary, but should be consistent. Be careful about over adjusting the scale to the point that tic marks are hard to understand

Large Particle Sorter

BioSorter

Union Biometrica

Kerckhoff B133

- Versatile instrument with 4 possible flow cells or FOCA:
  - FOCA 250
  - FOCA 500
  - FOCA 1000
  - FOCA 2000
- Large particle sorting - ~30µm to 800µm

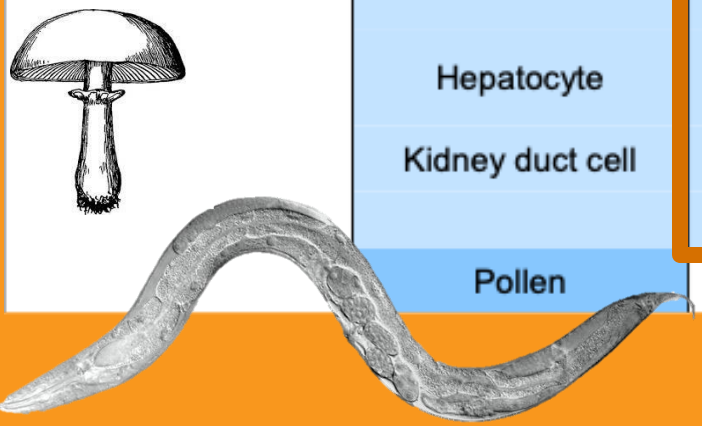
- Colinear lasers at 488nm and 561nm
- 3 Detectors:
  - Bandpass Filters: 512/25, 543/22, 615/25

- Sample deposition into a variety of plates and tubes including:
  - Culture plates (6, 12, 24, 48, or 96-well + custom)
  - PCR plates
  - 384-well plates
  - 1.5 mL, 5 mL, 15 mL and 50 mL tubes

- Sorting droplet is dispensed straight down for more precision into smaller welled plates

Applications

FOCA	250	500	1000	2000
Object Diameter Size Range	10-200 micron	10-350 micron	20-700 micron	40-1500 micron
		Beads (Alginate, Chemistry. Cells on Beads)		
Applications	*C.elegans*	*C.elegans*	Zooplankton	Zebrafish (embryos and larvae)
	Sea Urchin embryos	*Drosophila* (embryos and 1st instar larvae)	*Drosophila* (embryos, L1 and L2 instar larvae)	*Drosophila* (up to 3rd instar larvae)
		Mosquito (embryos and first instar larvae)	Mosquito (embryos and first, second and third instar larvae)	*Xenopus, Medaka, Daphnia*
	Stem cell	Pancreatic islet (mouse & rat)	Pancreatic islet (mouse, rat & human)	
	Hepatocyte	Embryoid body		
	Kidney duct cell	Adipocyte		
		Pancreatic duct cell		
	Pollen		Arabidopsis seed	

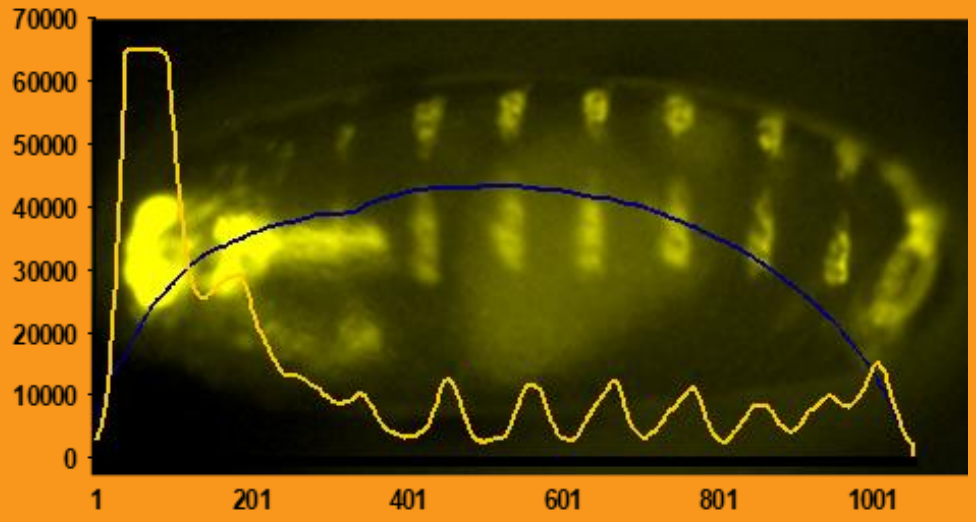


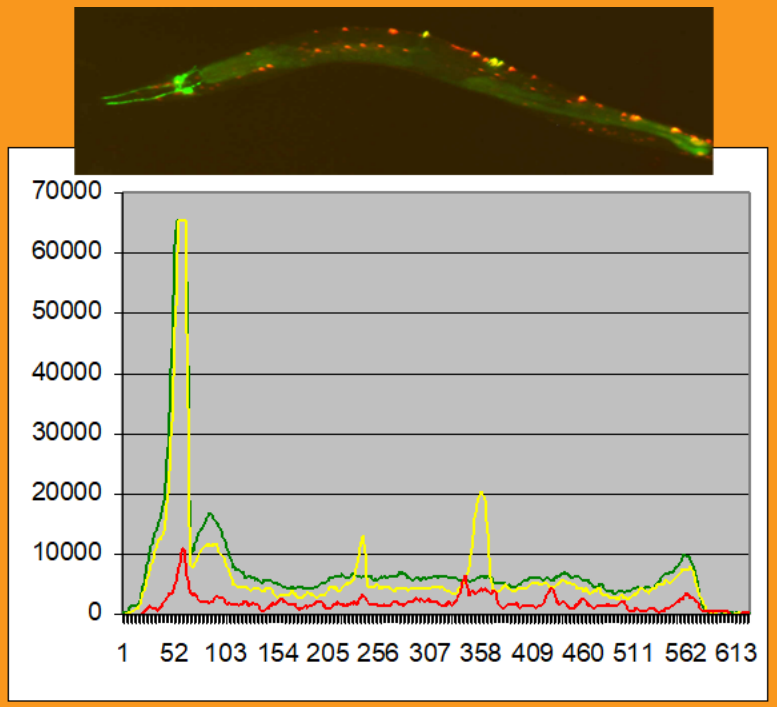
https://www.unionbio.com/applications/

BioSorter Special Features

- Straight down dispensing (only one population at a time)
- Very gentle sorting
- Recovery of unsorted ‘waste’ to rerun
- Plate/tray sorting
- Profiler

- Future?
  - More colors? Sterile enclosure?
  - Additional FOCA?





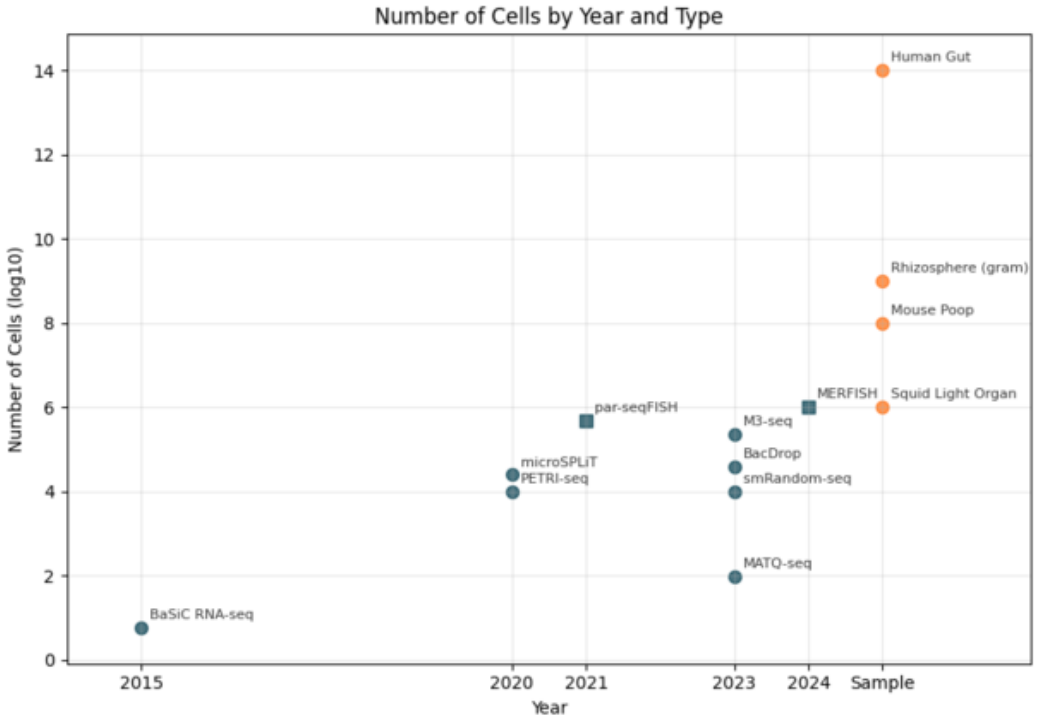
User Projects

- Sally Ireri, Ph.D. — Mengyi Cao Lab
- Vera Beilinson — Margaret McFall-Ngai/Pachter Labs
- Helena Awad — Linda Hsieh-Wilson Lab
- Amy Chow, Ph.D. — Mitchell Guttman Lab

Bacterial scRNA-seq isolated from host

Vera Beilinson
McFall-Ngai/Pachter Labs
Flow Cytometry Facility Lab Meeting
Oct 27, 2025

Bacterial scRNA-seq methods are increasing



NGS
Untargeted
Captures tmRNAs
Not all methods are reproducible (BacDrop)

Imaging
Targeted (price ↑ w/ # of genes, # of cells depe
on user)

Methods performed from *liquid culture* samples (no debris).
What about bacteria from a host?
Host debris is an issue

Goal: perform MATQ-seq on bacteria isolated from a host

Liquid Culture: Published for 2 species

Novel Method: Bacteria isolated from host

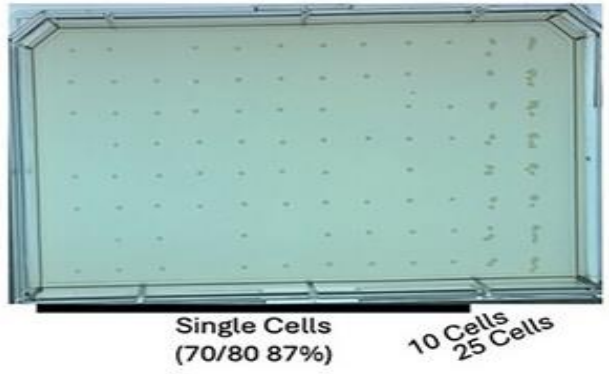
1. Cell sorting of bacteria
Flow cytometry (Method: Tijerina et al, 2025)

2. MATQ-seq (Homberger, 2023)

3. Sequencing

4. Analysis
Improved @ Pachter Lab (Oakes et al, ?)

Analysis



Success of bacterial single cell sorting @ Caltech w/ our “unoptimized method”

Goal: More accurate cells sorting

Previous sorting: size + presence of RFP plasmid → better to have counterstain to avoid debris in well

Goal: Identify a counterstain that is compatible for growth AND RNALater safe

→ Current no information online!!

→ RFP is RNALater safe (GFP and YFP is not)

Why:

→ Sorting live onto agar plates is a good sorting control to know success

→ For MATQ-seq it is ideal if the samples are fixed in RNALater prior to sorting since bacteria change transcription fast

Dye (1:250 and 0.2:250)	Liquid culture low OD	Liquid culture high OD	Isolated from host (squid)
SYTO9	No/minor growth	No/minor growth	No/minor growth
Bactoview Green	No growth	No growth	No growth
Calcein Violet	Growth	Growth	Growth
Hoescht	No growth	No growth	?

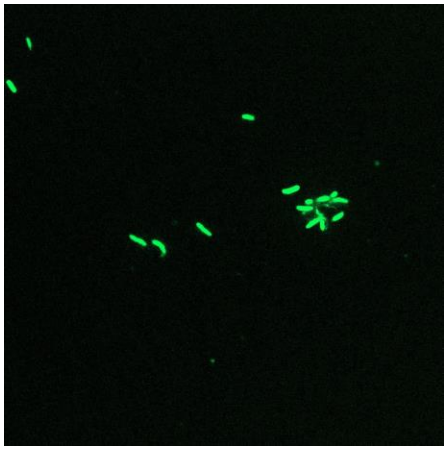
→ Calcein Violet is the only counterstain that allows for growth

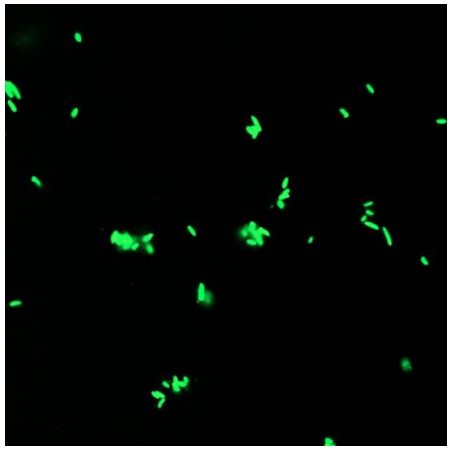
Which dyes are RNALater compatible

LIVE

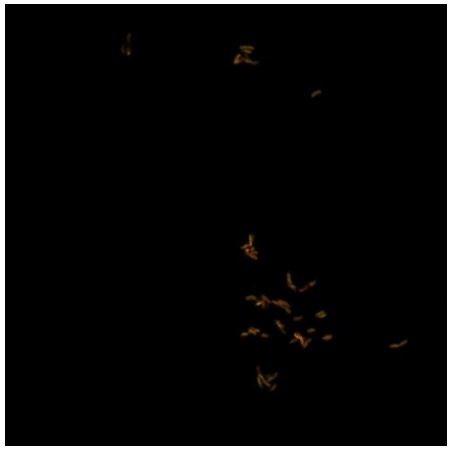
RNALater

WT RFP + Dye





WT RFP



→ **Syto9** is present in live and RNALater but Syto9 obliterated RFP

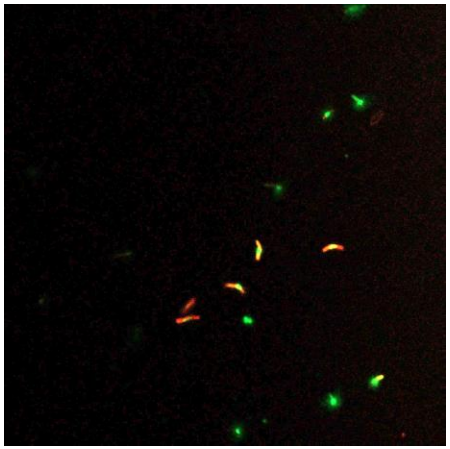
→ Some background in Syto9 channel

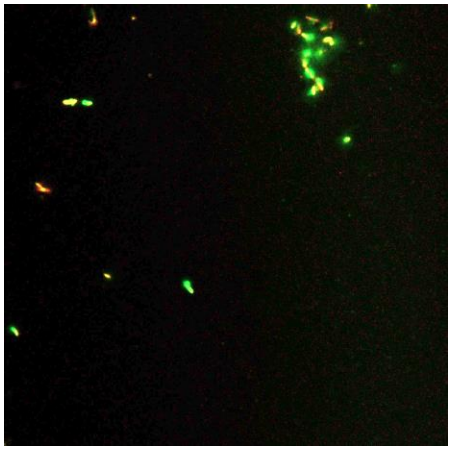
Which dyes are RNALater compatible

LIVE

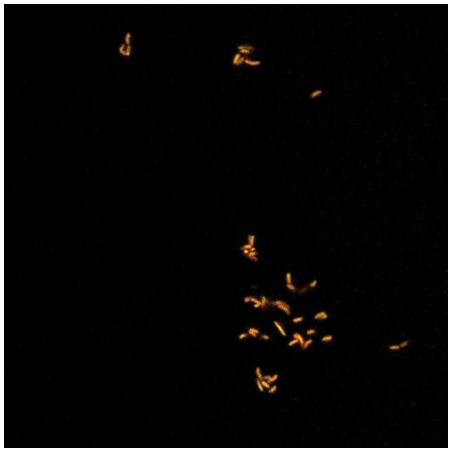
RNALater

WT RFP + Dye





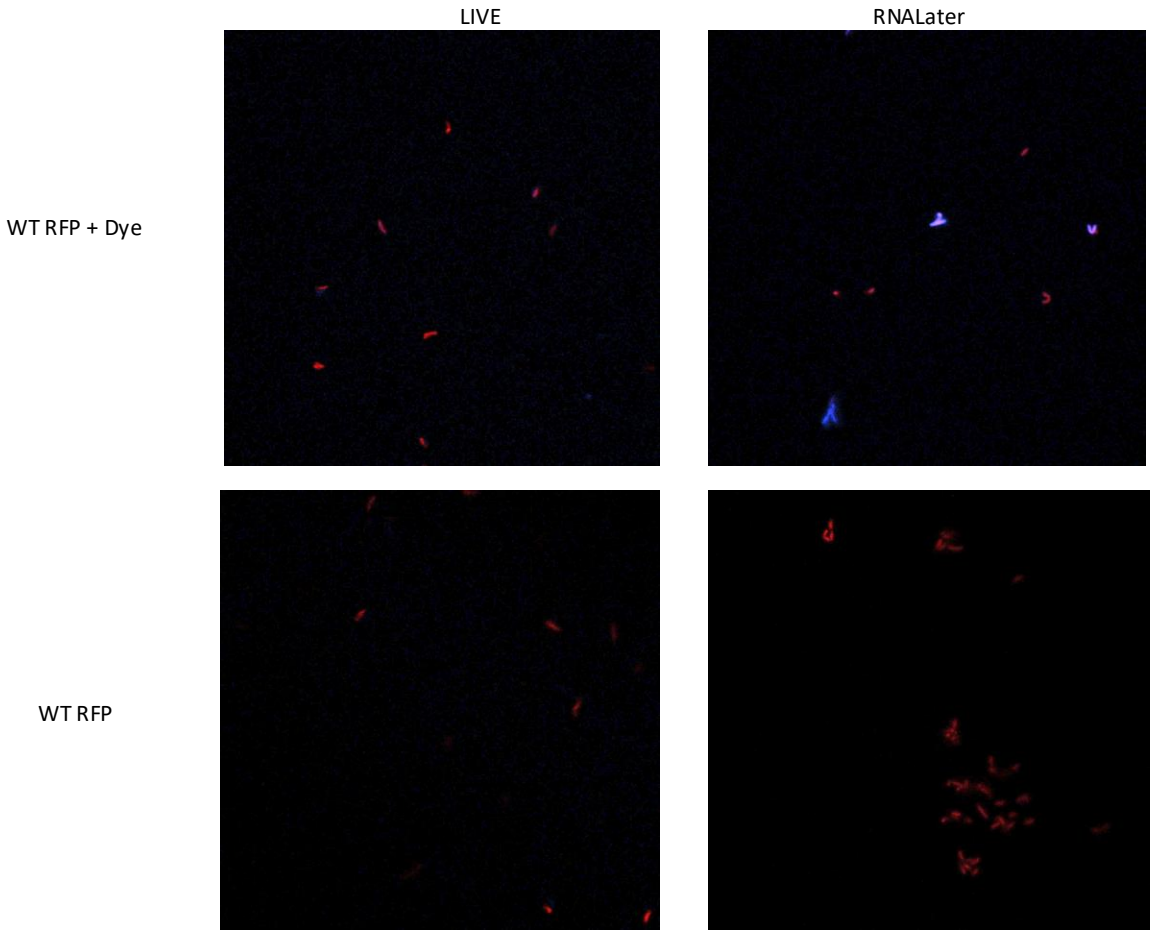
WT RFP



→ **Bactoview** is present in live and RNALater

→ Some background in Bactoview (FITC) channel

Which dyes are RNALater compatible



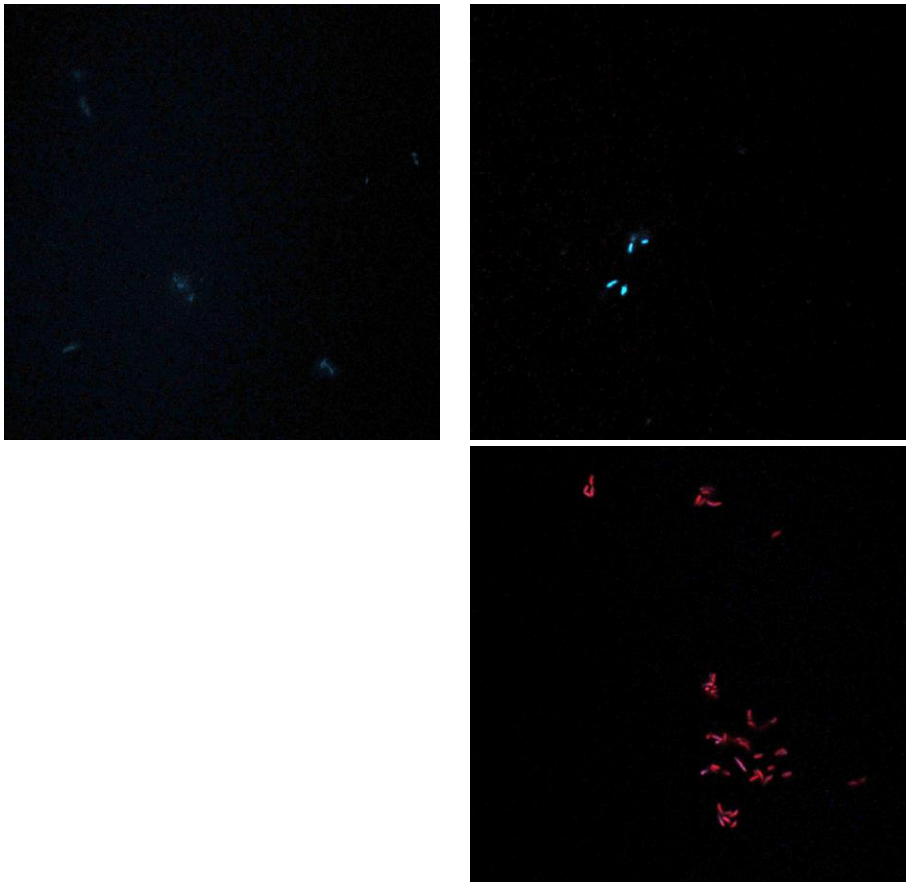
→ **Calcein Violet** is present in live and RNALater (possibly brighter in RNALater)
→ Little background in violet channel

Which dyes are RNALater compatible

LIVE

RNALater

WT RFP + Dye



WT RFP

→ **Hoescht** is present in live and RNALater but obliterates RFP signal

→ Little background in Hoescht channel

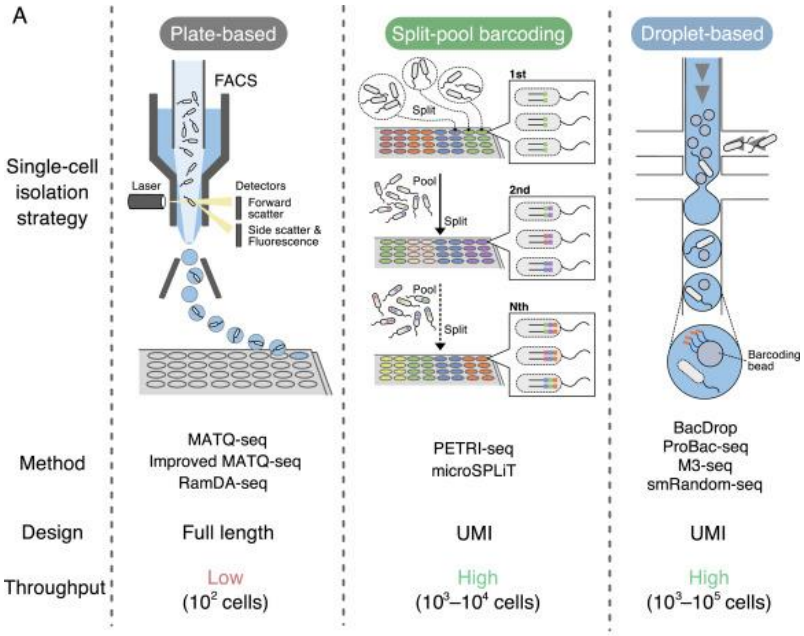
Conclusion

→ Calcein Violet is good counterstain allowing for growth
→ SYTO9, BactiView Green, Calcein Violet are RNALater fixable

Next steps:
Try sorting with calcein violet

Various methods

Highly reproducible



B

Method	Year	Features											
		Single-cell sorting strategy			cDNA generation & amplification		rRNA depeletion			Single-cell barcoding		
		FACS	Split-pool	Droplet	in situ generation	Random displacement amplification	RNaseH	Cas9	Other (Customed probe)	Split-pool	Droplet	Hybrid
PETRI-seq	2020		•		•					•		
MATQ-seq	2020	•										
microSPLiT	2021		•		•					•		
BacDrop	2023			•	•		•				•	•
ProBac-seq	2023			•	•				•		•	
M3-seq	2023			•	•		•				•	•
Improved MATQ-seq	2023	•						•				
smRandom-seq	2023			•	•			•			•	
RamDA-seq	2023	•				•		•				

Similar better method coming out soon!
Reproducible

Not reproducible

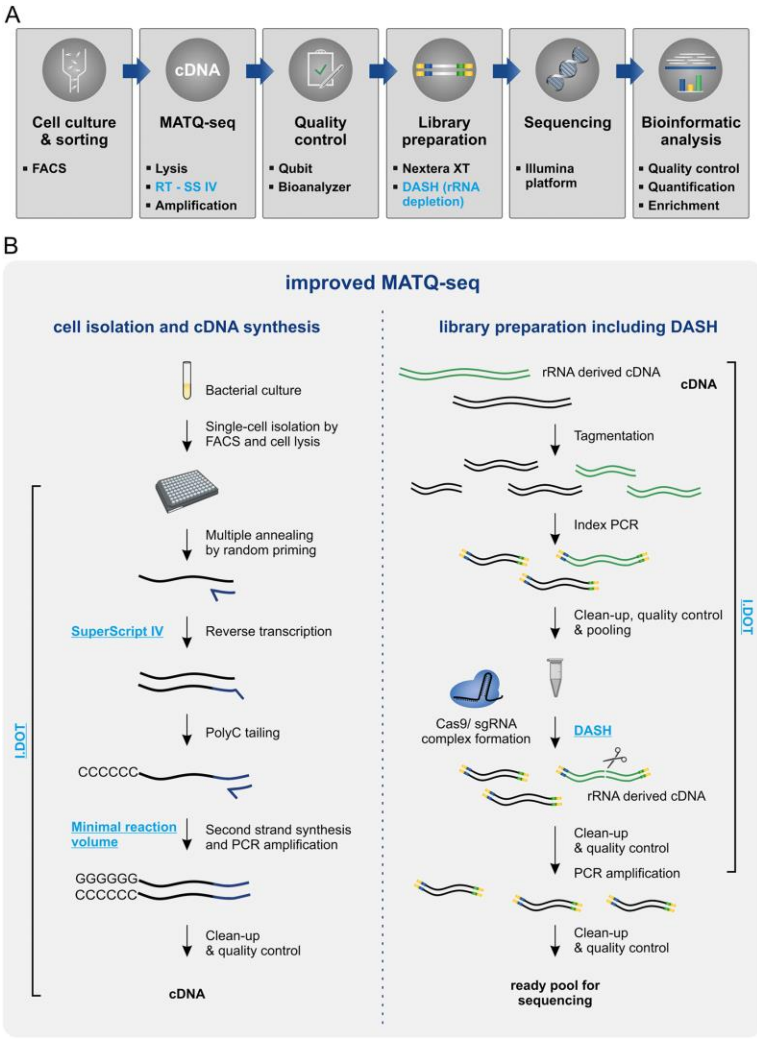
Reproducible

FACS sorting is vital regardless of method
→ remove dead cells + debris
→ ensures no bacteria sticking to each other*

*FACS sorter w/ “imager” is recommend to visualize the single cell

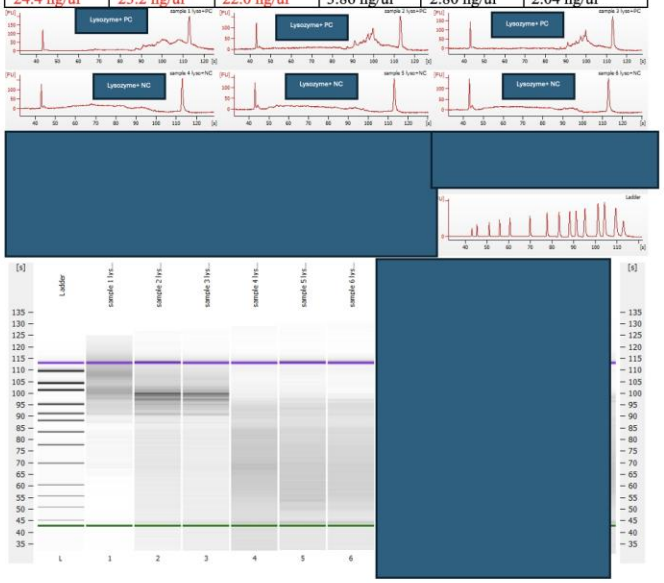
Most methods are similar-
just depends if in well or within cell

Nishimura 2025



Homberger et al 2023

Sample 1: RNA+ Lysozyme + 16.2 ng/ul	Sample 2: RNA+ Lysozyme + 9.64 ng/ul	Sample 3: RNA+ Lysozyme + 11.6 ng/ul	Sample 4: NC Lysozyme + 3.76 ng/ul	Sample 5: NC Lysozyme + 2.04 ng/ul	Sample 6: NC Lysozyme + 2.64 ng/ul
Sample 7: RNA+ Lysozyme – 24.4 ng/ul	Sample 8: RNA+ Lysozyme – 23.2 ng/ul	Sample 9: RNA+ Lysozyme – 22.0 ng/ul	Sample 10: NC Lysozyme – 3.86 ng/ul	Sample 11: NC Lysozyme – 2.80 ng/ul	Sample 12: NC Lysozyme – 2.04 ng/ul

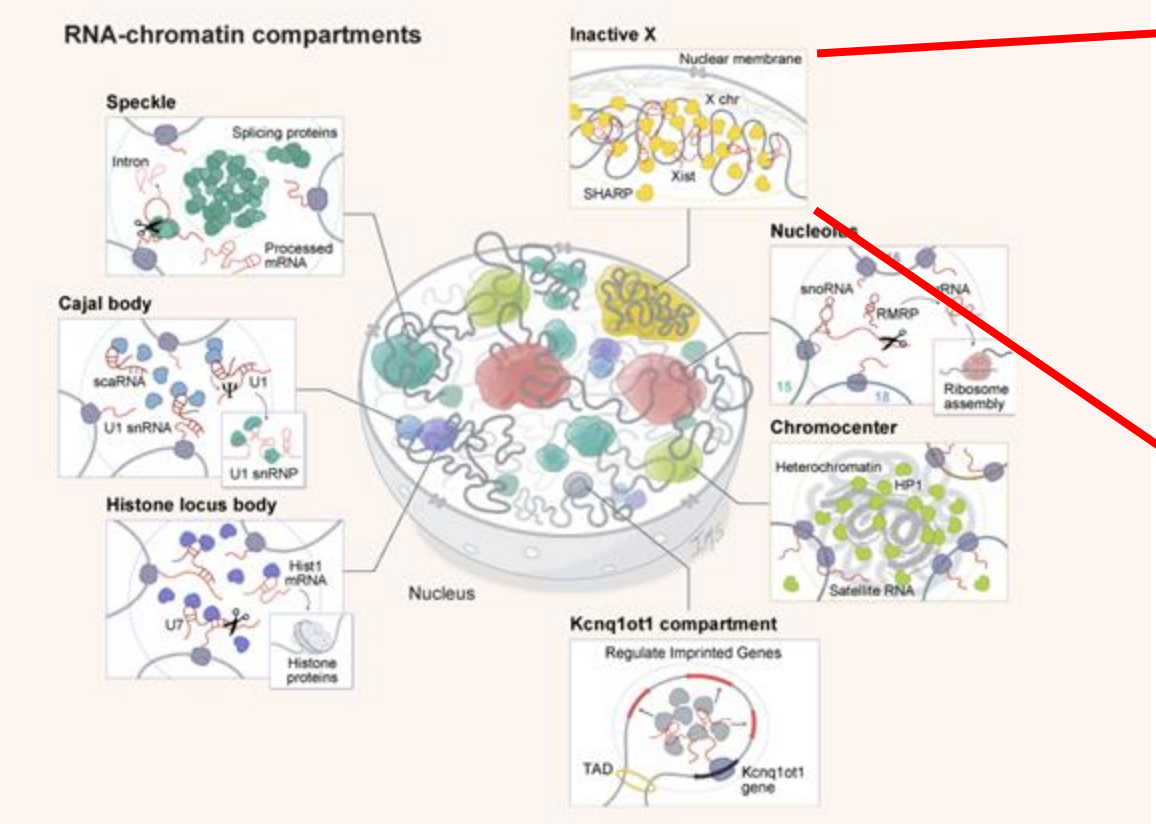


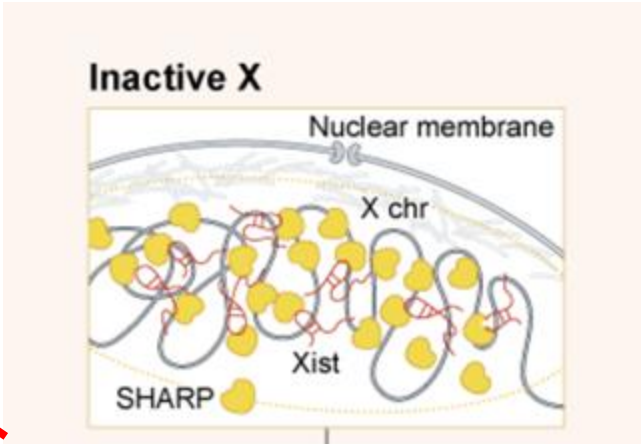
Vera – success @ Caltech. cDNA(no DASH). NC are low, PC are within range w/ expected peak shape.
Critical to do in PCR/clean hood.

PrimeFlow RNA

Amy Chow, Ph.D.
Guttman Lab

Guttman lab research focus

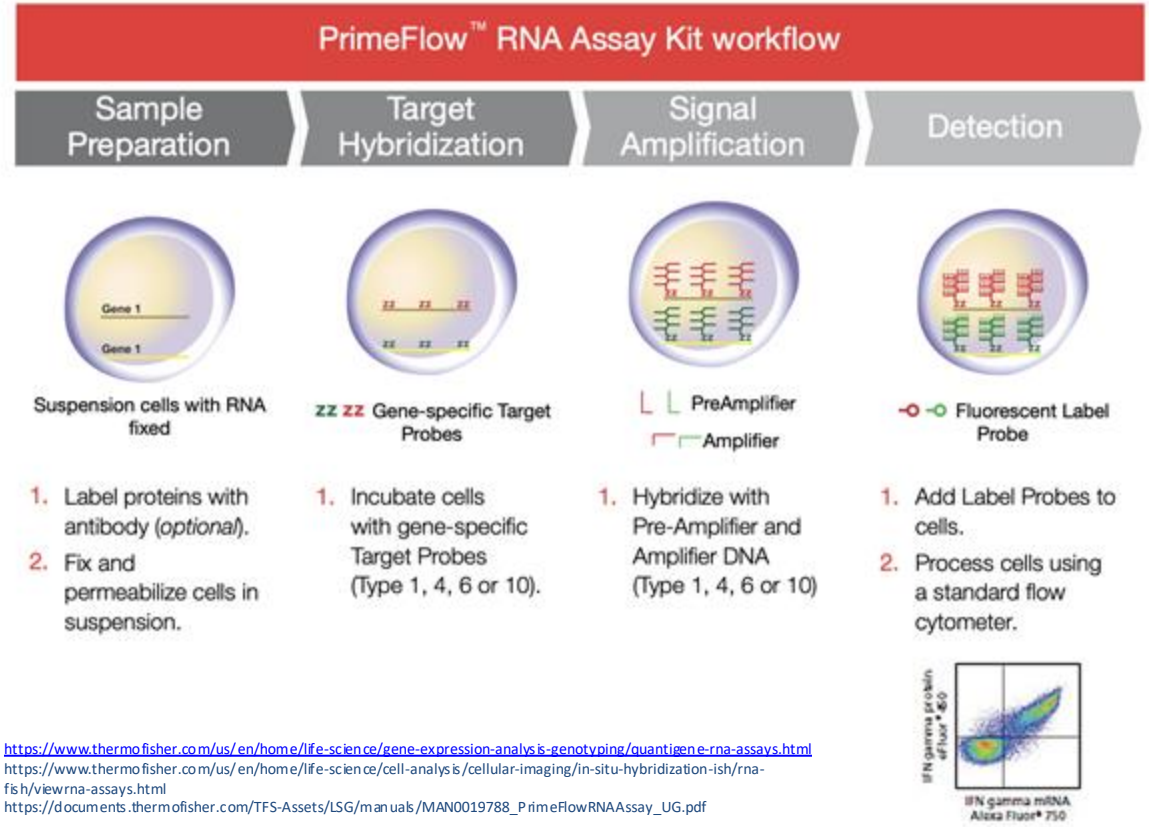


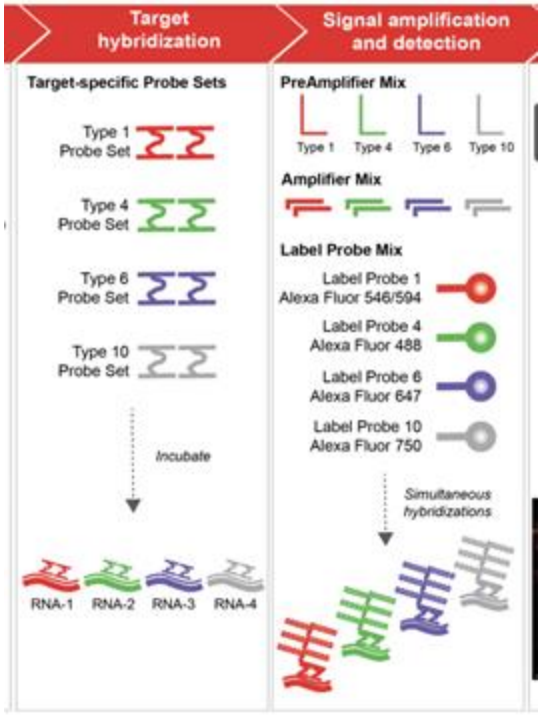


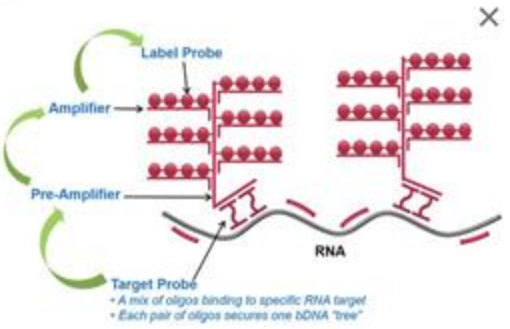
Understanding the role of ncRNAs in organizing chromatin architecture and the impacts on gene expression

https://guttmanlab.caltech.edu/research/

PrimeFlow RNA principle

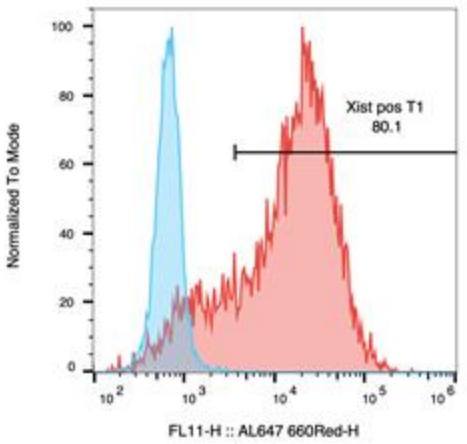






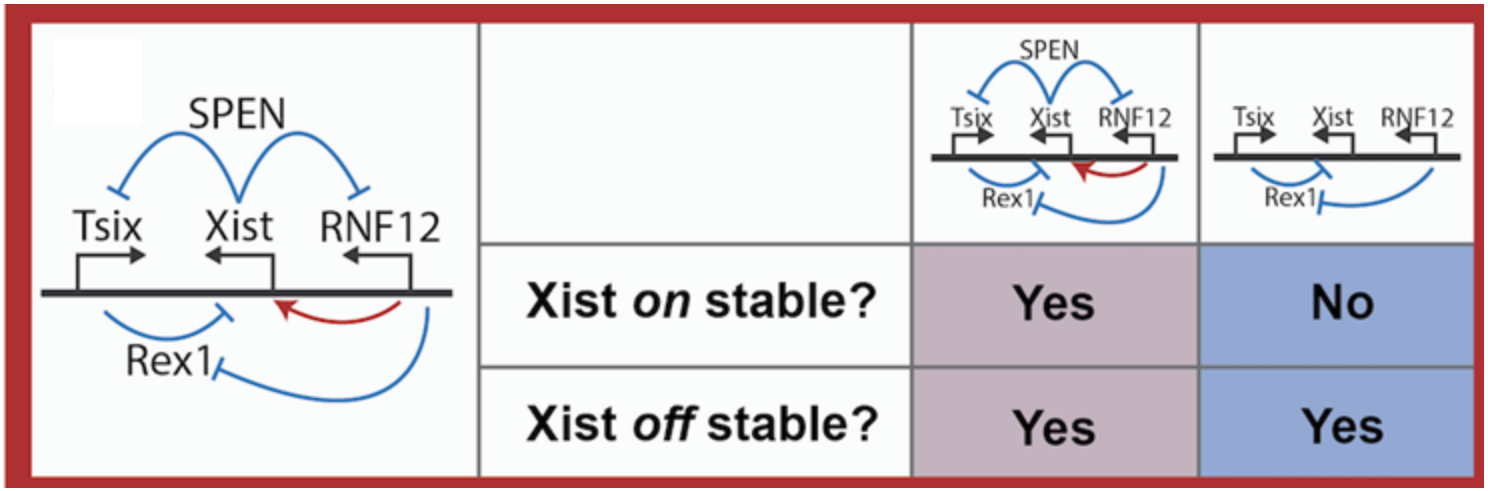
https://www.thermofisher.com/us/en/home/life-science/gene-expression-analysis-genotyping/quantigene-rna-assays.html
https://www.thermofisher.com/us/en/home/life-science/cell-analysis/cellular-imaging/in-situ-hybridization-ish/rna-fish/viewrna-assays.html
https://documents.thermofisher.com/TFS-Assets/LSG/manuals/MAN0019788_PrimeFlowRNAAssay_UG.pdf

Our results and future plans



Negative controls critical for determining background

CRISPRi screen to find regulators such as Rex1



Please give us your Feedback!

Feedback survey →

Future needs questions:

- Spectral instruments?
- Imaging instruments?
- Booking sorting directly?

Other needs/questions?

Please scan the QR code to rate your experience with the Facility and give us some feedback.