Selected Topics in Electrical Engineering: Flow Cytometry Data Analysis

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Outline

- Experimental design and fluorescence quantitation
  - Data acquisition ground rules
  - Choosing a fluorochrome
  - Choosing an antibody
  - Negative controls
  - Fluorescence quantitation

Experimental Design for Flow Analysis

- A flow cytometer is a delicate instrument
  - Many parameters determine the acquired data
    - Sample preparation
    - Fluorescence staining
    - Flow analysis
  - The choices of these parameters – i.e. the settings – must allow for an adequate analysis of the biological question of interest

→ Everything depends on the biological question of interest

Experimental Design for Flow Analysis

- Decisions need to be made concerning
  - The number of events (cells and other particles) to be acquired
  - The rate of sample acquisition
  - The optimal fluorochromes
  - The monoclonal antibodies
- The options are:
  - Run a preliminary analysis and see how this works out with the first-choice selections
  - Try to predict the outcomes for the various alternatives and take action accordingly

Determining the Number of Events/Cells to be Acquired

- Flow experiments are run to gather information on a specific cell type
- The number of acquired events has to be high enough to provide a sufficient number of cells of interest
  - for viable statistical analysis

➔ None of that "… number of events acquired as list mode data" stuff!!

- Example: Rare-event analysis
  - Stem cells are very rare among the blood cells
    - Hematopoietic progenitors
  - Consist about 0.2% of all events
    - 10000 events would contain about 20 cells
    - 100000 events would contain about 200 cells
    - …

Determining the Number of Events/Cells to be Acquired

- Suggested practice:
  - Determine the expected frequency of the cell type of interest
    - The frequency of the rarest, if multiple cell types are of interest
  - Carry on with event acquisition until a sufficient expected number is reached
- Remarks:
  - Cell percentages can differ dramatically based on the condition of the person
    - Age/gender
    - Healthy vs. suffering from a disease
    - Undergoing treatment or medication
    - …

Determining the Number of Events/Cells to be Acquired

- Alternatively:
  - The experiment can be carried on until a fixed number of the rarest cell type has been acquired
    - A region of acceptance is defined in terms of the measured flow parameters
      - Forward scatter, side scatter, fluorescence staining
    - Acquisition stops when a given number of events in the region have been counted
      - The region of acceptance to define the parameters associated with the rarest cell type of interest

Adjusting the Rate of Sample Acquisition

- Response time of the electronic circuitry imposes a limit on the number of events that can be recorded in a second
  - About 3000 events per second
- Sufficient quantities of rare cells requires recording many millions of events

➔ May amount to a substantial amount of time

  - Problematic especially if the fluorescent staining is unstable

- A way out is to adjust the event detection threshold to a suitable level
  - Flow cytometers apply event detection
    - By thresholding (on the forward scatter signal)
    - To distinguish signals from actual events against noise
  - Adjusting the threshold slightly lower than the expected value from the cell of interest allows
    - Not activating the device electronics for the other events
    - Running the instrument at a higher rate

Selecting the Right Fluorochromes

- All flow cytometers are equipped with some choice of optical devices
  - Lasers, beam splitters, dichroic mirrors, filters, detectors
- The choice of fluochromes has to abide with the instrument specifications above all else
- Beyond this basic constraint, other factors need to be considered when choosing fluorochromes, especially for high color experiments
  - Excitation/emission specifications
    - Emission peaks cannot overlap in simultaneous use
  - Susceptibility to quenching
  - Quantum efficiency in varying temperature and pH
  - …

  (see the long list at http://flowcyt.salk.edu/fluo.html)

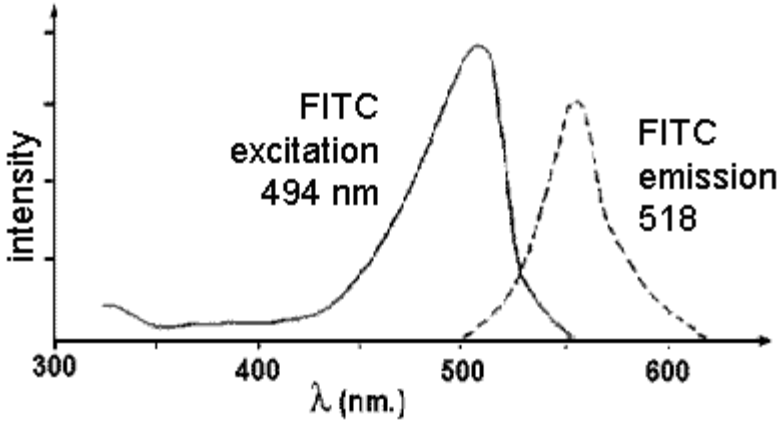
Probe	Ex (nm)	Em (nm)	MW	Notes
Reactive and conjugated probes				
Hydroxycoumarin	325	386	331	Succinimidyl ester
Aminocoumarin	350	445	330	Succinimidyl ester
Methoxycoumarin	360	410	317	Succinimidyl ester
Cascade Blue	(375);401	423	596	Hydrazide
Pacific Blue	403	455	406	Maleimide
Pacific Orange	403	551		
Lucifer yellow	425	528		
NBD	466	539	294	NBD-X
R-Phycoerythrin (PE)	480;565	578	240 k	
PE-Cy5 conjugates	480;565;650	670		*aka* Cychrome, R670, Tri-Color, Quantum Red
PE-Cy7 conjugates	480;565;743	767		
Red 613	480;565	613		PE-Texas Red
PerCP	490	675		Peridinin chlorphyll protein
TruRed	490,675	695		PerCP-Cy5.5 conjugate
FluorX	494	520	587	(GE Healthcare)
Fluorescein	495	519	389	FITC; pH sensitive
BODIPY-FL	503	512		
TRITC	547	572	444	TRITC
X-Rhodamine	570	576	548	XRITC
Lissamine Rhodamine B	570	590		
Texas Red	589	615	625	Sulfonyl chloride
Allophycocyanin (APC)	650	660	104 k	
APC-Cy7 conjugates	650;755	767		PharRed

Source: http://flowcyt.salk.edu/fluo.html

Selecting the Right Fluorochromes

- Fluorescein isothiocyanate (FITC):
  - Excitation/emission at 495 / 521 nm
    - Relatively poorly excited by 488 nm lasers
  - The first fluorochrome to be conjugated to a protein
    - Available commercially as pre-conjugated to various monoclonal antibodies
  - Rapidly quenched in low pH environments
  - Long emission spectrum
    - Problematic when used in conjunction with other fluorochromes excited at 488 nm
  - Poor choice for studying weakly expressed antigens

Fluorescein	495	519	389	FITC

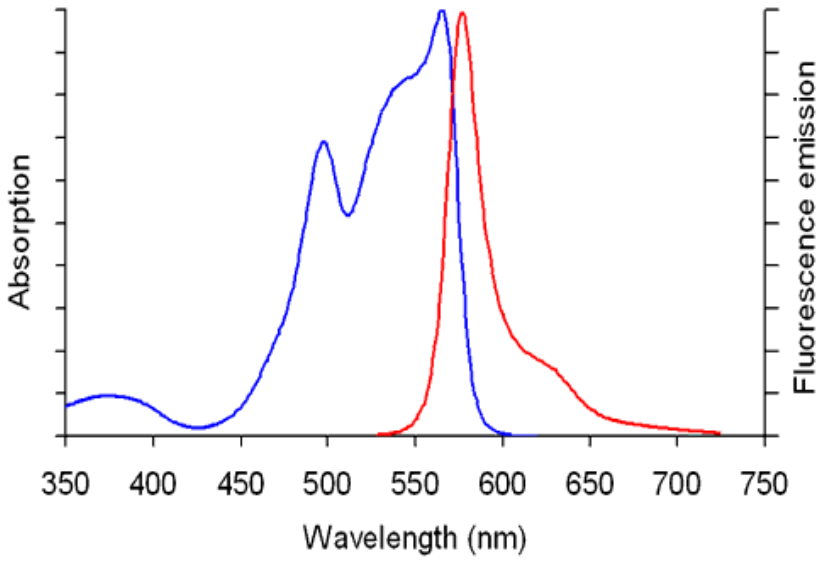


Source: http://web.uvic.ca/ail/techniques/epi-fluorescence.html

Selecting the Right Fluorochromes

- Phycoerythrin (PE):
  - Composed of a protein part covalently binding chromophores called phycobilins
    - **R-PE**, B-PE
  - Optimally excited by green lasers
  - Extremely photo-stable
  - Possesses high quantum efficiency
    - High signal-to-noise ratio → suitable for weakly expressed antigens
  - Very large size
    - Not suitable for intracellular staining applications

R-Phycoerythrin	480;565	578	240 k	PE

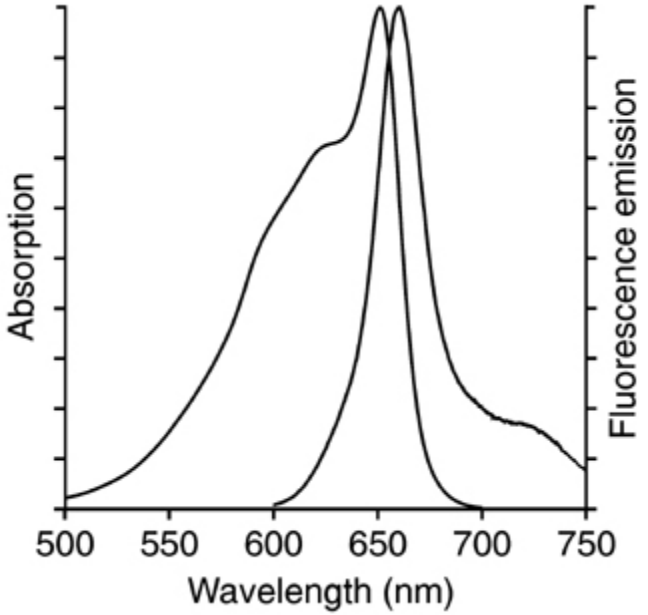


Source:
http://www.invitrogen.com/site/us/en/home/support/Product-Technical-Resources/Product-Spectra.801ph75.html

Selecting the Right Fluorochromes

- Allophycocyanin (APC):
  - Also a phycobiliprotein
  - Photo stable
  - Good quantum efficiency
  - Relatively easy to conjugate to proteins
  - Very bright
    - Suitable for weakly expressed antigens

Allophycocyanin	650	660	104 k	APC



Source:
http://products.invitrogen.com/ivgn/product/A10688?ICID=search-product

Selecting the Right Fluorochromes

- PE and APC tandem dyes:
  - Phycobiliproteins are good donors to be used in tandem dies
    - Peridinin chlorophyll protein – PerCP (the only naturally occurring tandem fluorochrome)
    - PE/Texas Red
    - PE/Cy5, APC/Cy5
    - PE/Cy7, APC/Cy7
    - …
  - As the quantum efficiency is reduced, tandem dyes not suitable for weakly expressed antigens

Selecting the Proper Antibody

- Monoclonal antibodies carry out specific binding to target molecules - antigens
- Different monoclonal antibodies (mAbs) can be specific to the same antigen
- But different mAbs may bind to different regions of the target antigen
  - called epitopes
- This may cause the results to be epitope-specific
  - Sample preparation can affect the affinities of the mAbs to their respective epitopes differently
  - The results acquired from different experiments using mAbs targeting the different epitopes of the same antigen may not be comparable
- This calls for maintaining the same choice of mAbs for all experiments related to the same study

Clusters of Differentiation

- One of the main application areas of multicolor flow cytometry is the differentiation between the different types of leukocytes
- The leukocyte antigens that act as targets for the mAbs conjugated to fluorochromes for fluorescent staining are identified through a protocol called

clusters of differentiation

  - Intended for the classification of different mAbs generated by different research groups
  - Commonly used as cell markers
    - That associate the cells possessing them with specific functions

Clusters of Differentiation

- The classification is generated by Human Cell Differentiation Molecules organization
  - Periodically organizes the Human Leucocyte Differentiation Antigens Workshop
  - Curates a database of CDs including their characterizations
    - mAbs, epitopes, …
    - http://www.hcdm.org/
- As the CDs are also proteins, a detailed documentation and list is also provided by the UniProt protein database
  - http://www.uniprot.org/docs/cdlist

Negative Controls

- Detection of positively stained events require a threshold
  - To distinguish positive events from those that exhibit the background fluorescence level of mAb binding
- In theory, this can be accomplished by running the flow cytometer on isotype-matched samples
  - Identical samples but without the fluorochrome
  - To determine the extent of background non-specific binding and autofluorescence
- The usage, however, is problematic:
  - The degree of non-specific binding can differ for the same isotype due to other factors
  - The fluorochrome to protein ratio (F/P ratio) must be matched for the mAb
  - …
- Thus, the use of negative controls require careful preparation

Negative Controls

- Negative controls are feasible for surface-staining
  - In protocols that target antigens on the cell surface
  - Provided that the previously raised issues are addressed adequately
    - the isotype control antibody matching the primary antibody's host species and isotype
    - using the same antibody concentration for both the isotype control and the primary antibody
- Negative controls are not feasible for intracellular staining
  - Staining patterns get affected by the stimulated cells

Measurement of Fluorescence Intensity

- The fluorescence intensity measured from each event is related to
  - The number of target molecules present at the cell
  - The number of fluorochromes bound to each target molecule
  - The fluorescence yield associated with each fluorochrome
    - Optimal excitation through the absorption spectrum
    - Optimal detection of the emitted light through the emission spectrum
    - Quantum efficiency
- Generally, the measured fluorescence intensity relates to three different types
  - Relative intensity
  - Semi-quantitative intensity
  - Quantitative intensity

Measurement of Fluorescence Intensity

- Relative fluorescence intensity:
  - The median intensity of population A is compared to the median intensity of the population B
    - Median intensities to be computed on log-normal distributions
      - Measured intensity values are taken to the natural logarithm
  - The percent relative intensity is calculated via

    100 ×(median intensity A – median intensity B) / (median intensity B)
    - assuming the median intensity of the population A if higher
  - Notes:
    - The experiments for the two populations need to be run in succession at the same flow cytometer
      - to remove any undesired systematic changes

Measurement of Fluorescence Intensity

- Semi-quantitative fluorescence intensity:
  - To evaluate the fluorescence intensities observed in an experiment, a reference is needed
  - Such a reference can be obtained by latex beads acting as fluorescent standards
  - Typically,
    - A collection of beads contains five or six different populations differing from each other in nominal fluorescence intensity
    - The collection is run through the flow cytometer
    - The median fluorescence intensities of each population is calculated over the histogram
      - Over intensities taken to their natural logarithm
    - The median fluorescence values are linked to the known fluorescent values provided by the bead manufacturer
      - Associating each fluorescence intensity to a specific number of fluorochrome/bead value taken to the natural logarithm
  - This allows some form of standardization
    - Though the fluorochrome/bead specifications can differ between the different manufacturers

Measurement of Fluorescence Intensity

- Quantitative fluorescence intensity:
  - Beads coated with **known numbers** of a specific antigen can be conjugated with the corresponding mAb conjugated to a fluorescent dye
    - This process mirrors the sample preparation for a regular flow experiment
  - Through a similar analysis to the one before, a relationship between the
    - Known numbers of fluorochrome-conjugated mAbs and
    - The observed fluorescence intensity

    can be identified
  - Notes:
    - The affinity of the binding to the antigen on the bead may not necessarily equal the affinity of the binding to the antigen of the cells of interest
    - The antibody needs to be specific only to one epitope of the target antigen

Summary

- The details for setting up a successful flow experiment are abound
- Choices regarding the various settings have to be made in an informed manner depending on
  - The biological question under investigation
  - The information to be extracted
- Errors in the experimental setup can easily render the acquired data unusable