Selected Topics in Electrical Engineering: Flow Cytometry Data Analysis

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Outline

- Fluorochromes and fluorescence
  - Light emission from materials
  - Fluorescent staining

Light emission from materials

- Incandescence
  - Light emission from hot surfaces due to heat
- Luminescence
  - Light emission without any regard to heat energy
    - An electron gets raised to a higher energy state
    - As it decays to its ground state, light is raised with the corresponding amount of energy/frequency
  - Several distinct types exist:
    - Chemiluminescence (bioluminescence if it occurs in living cells)
      - Electron excitation due to a chemical reaction
    - Photoluminescence
      - Electron excitation due to absorbed light
      - Occurs in the form of fluorescence or phosphorescence

Light emission from materials

- Light is emitted at discrete energy levels
  - Light energy is measured by the Planck-Einstein equation:

$$E = h \times \nu$$

where

E denotes the light energy,

h denotes Planck's constant at $6.626\ 10^{-34}$ Joules-second, and

ν denotes its frequency

  - In vacuum, this equation becomes

$$E = h \times \left(\frac{c}{\lambda}\right)$$

Light emission from materials

- Thus, the energy of a single photon at frequency
  - 360nm is 5.5178 10^{-19} Joules
  - 408nm is 4.8687 10^{-19} Joules
  - 488nm is 4.0705 10^{-19} Joules
  - 595nm is 3.3385 10^{-19} Joules
  - 633nm is 3.1381 10^{-19} Joules
- At the interrogation point, a cell is illuminated for 5 10^{-5} – 5 10^{-6} seconds

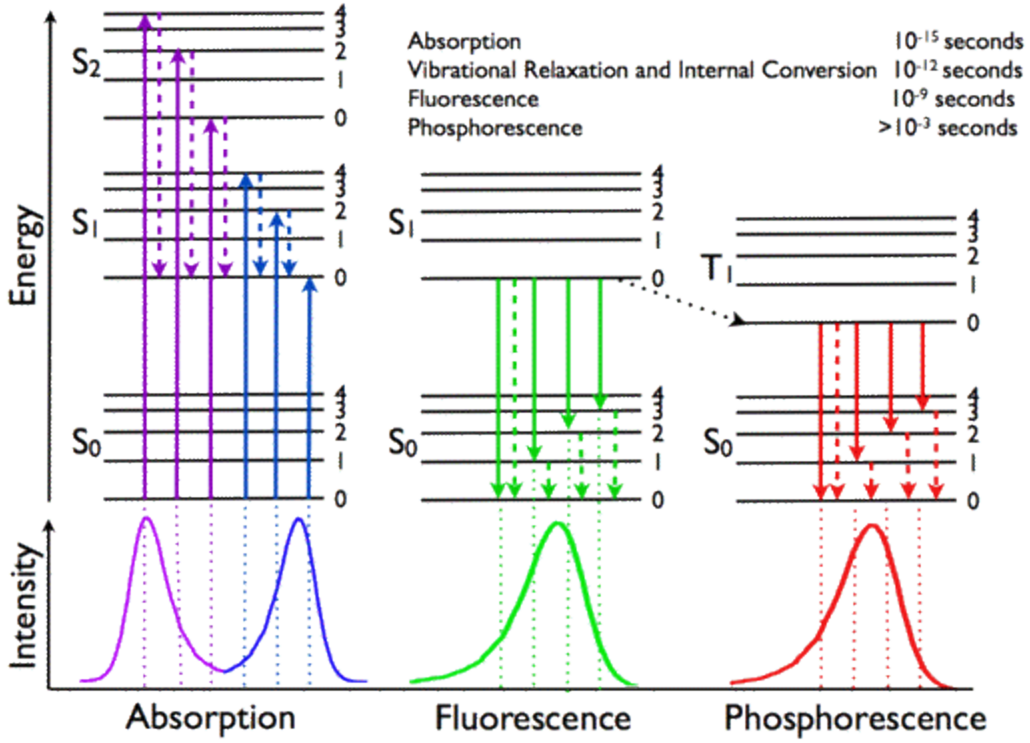
➔ It will receive 10^{10} – 10^{11} photons

  - Depending on the cross-sectional area of the cell and the beam

Light emission from materials

- Fluorescence is caused by
  - Light absorption knocking an electron off its ground state to a state with higher energy
    - By a photon possessing the “correct” energy/frequency
  - When the electron returns to its ground state, the “stored” energy is released as another photon with the corresponding energy/frequency
- Remarks:
  - The absorption spectrum of an atom is discrete
    - Peaks at the frequencies/wavelengths leading to absorption
    - For hydrogen, for instance, these peaks are at 102.6, 121.6, 486.9, and 657.3 nm
  - The situation is more complicated for large molecules
    - A large number of interactions between the different atoms are possible
    - These interactions possess a variety of differing characteristics

Energy bands in excitation and emission



A Jablonski diagram

Source: http://www.photobiology.info/Visser-Rolinski.html

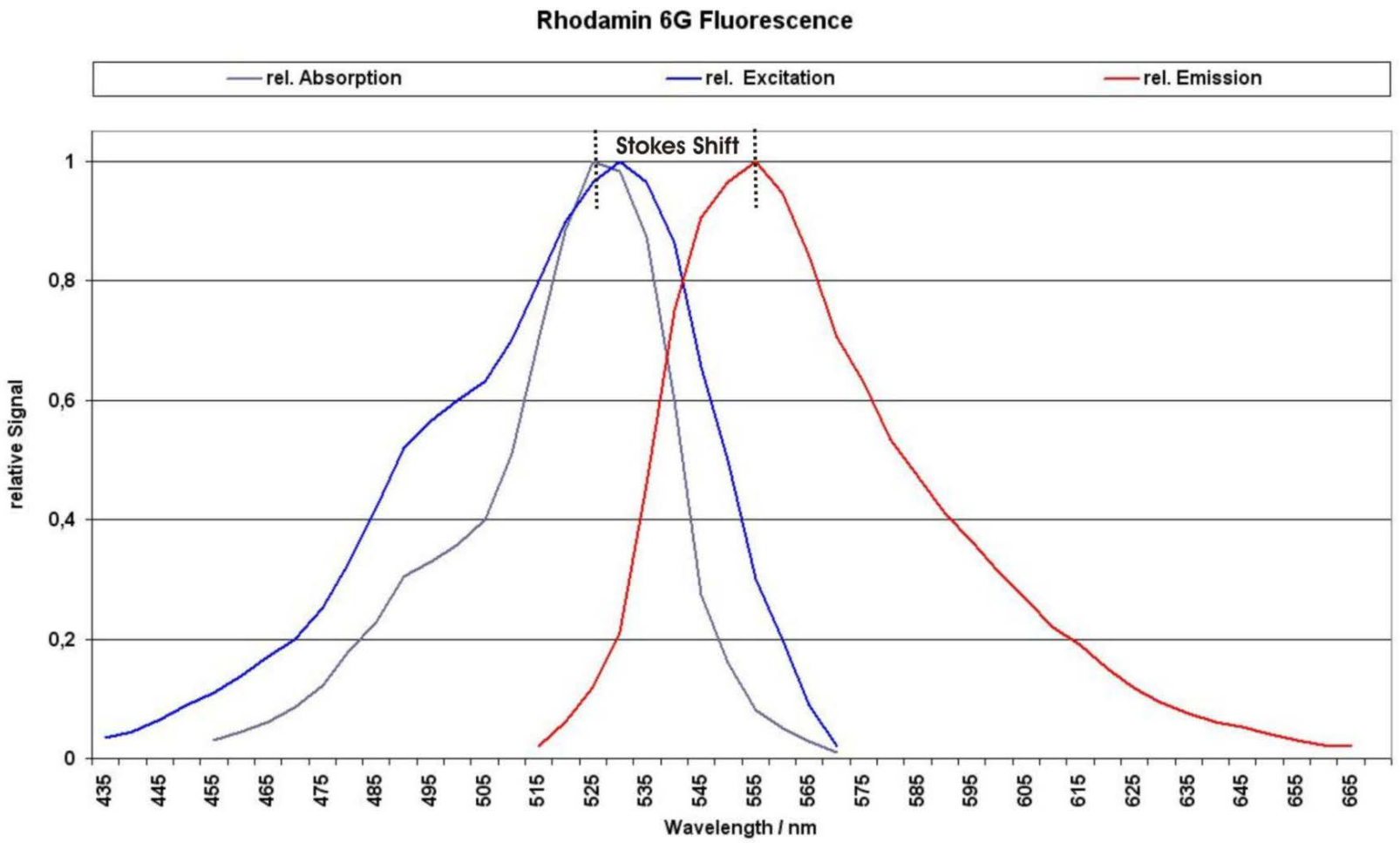
Stoke's shift

- The energy of an excited electron is reduced due to several non-fluorescent events
  - Vibrational relaxation within electronic excitation states
  - Internal conversion between higher excitation states
  - External quenching (i.e. resonance)
  - Intersystem crossing between the singlet energy state to the triplet state
- The net result is a marked reduction of the emitted photon energy

  → reduced frequency

  → increased wavelength

Stoke's shift



Fluorescence intensity

- The intensity of a fluorochrome is critical in practical applications
  - Impacts the detection sensitivity
- Governed by the expression

$$F = I_o \epsilon [C] x \phi$$

where

- F is the intensity of light emitted
- I_o is the incident intensity
- ϵ is the molar extinction coefficient (of the fluorochrome)
- $[C]$ is the molar concentration (of the fluorochrome)
- x is the path length
- ϕ is the quantum yield

The quantum yield

- The quantum yield measures the ratio of emitted photons to the absorbed photons
- Due to various factors contributing to energy loss following excitation, this ratio is always smaller than 1
  - internal conversion
  - quenching
  - intersystem crossing

Remarks

- In fluorescence, the process of excitation followed by emission can be repeated many times
  - In contrast to chemiluminescence where the substrate is chemically altered once and for all
- Fluorescence is eventually lost due to photobleaching
  - Especially in high intensity illumination
  - Reactions between the excited fluorochromes and nearby oxygen produces singlet oxygen
  - The result is the irreversible oxidation of the fluorochromes
  - The phenomenon, however, is much more critical in fluorescence microscopy applications

Fluorescent staining

- Fluorescent staining can occur in several ways
  - Some dyes have a natural affinity to certain substances
    - Lipophilic dyes accumulate in membranes
    - Reactive dyes bind proteins
    - …
  - Often, fluorochromes are conjugated to ligands that bind specifically to their receptors
    - Antibodies
  - Some fluorochromes exhibit different quantum yields when they are bound to a particular substance
    - The fluorescence of ethidium bromide increases 100 times when bound to DNA

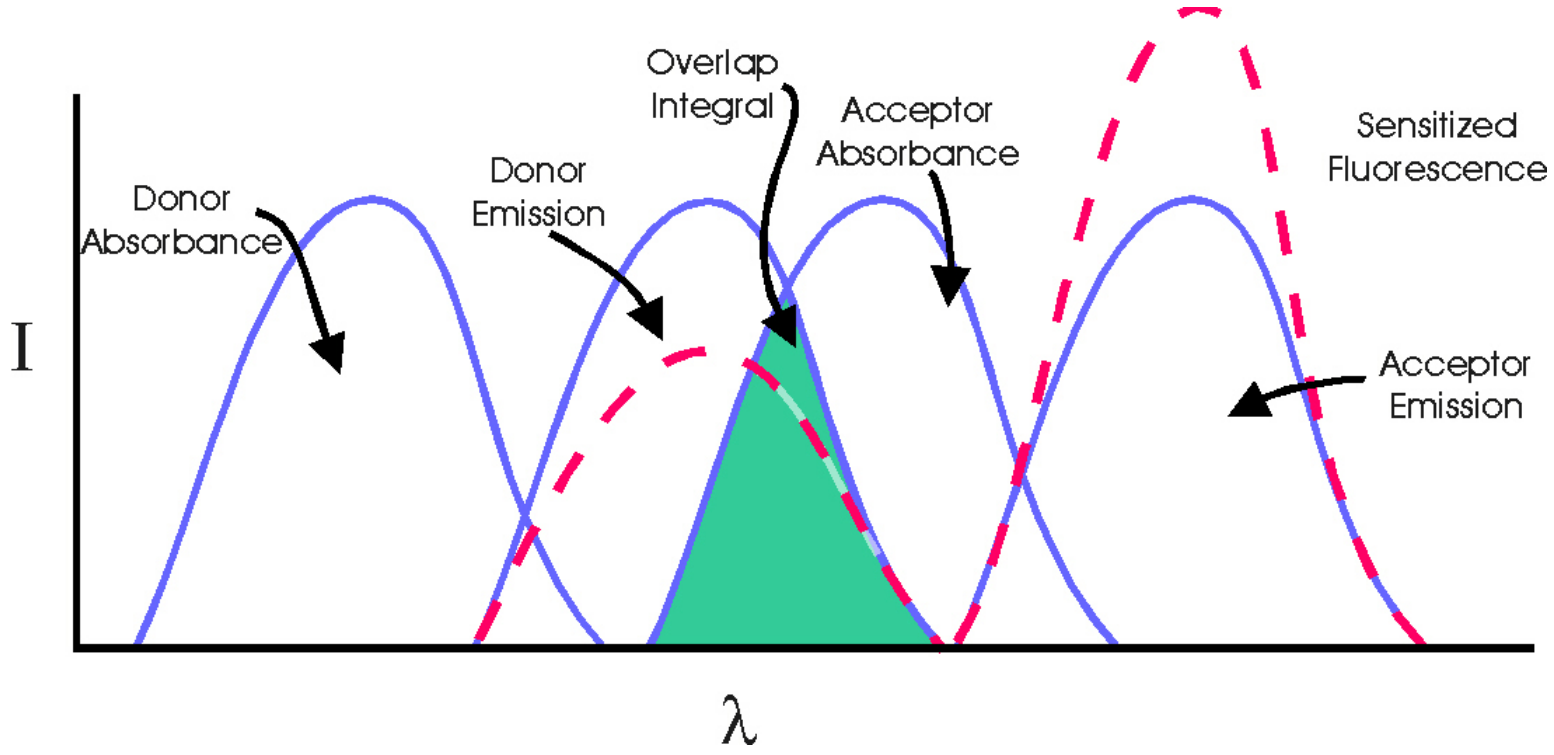
Cellular autofluorescence

- Certain substances in cells have fluorescent properties
  - pryidines
    - NAD, NADH, NADP, NADPH]
  - flavins
    - FAD
    - FMN
  - porphyrins
    - Protoporphyrin
- Generally, these molecules are:
  - excitable by light sources in the higher frequency ranges (blue) and
  - fluoresce in the lower frequency ranges (green-orange)
- Autofluorescence in blood leukocytes exhiibits very similar excitation and emission spectra
  - But the emission intensity is dramatically lower → weak light emission
- Fluorescence intensity from neutrophils and eosinophils, however, is higher and can interfere with signal at the green frequencies
  - Fluorescein
- This may require using fluorochromes with emission spectra at higher wavelengths (>600nm)

Fluorescence resonance energy transfer (FRET)

- When two different fluorochromes are in close proximity, their electron bands interact
- This interaction occurs through coupled oscillator mechanisms ➔ resonance
  - The fluorochrome with the lower excitation wavelength/higher frequency is called the donor
  - The other is called the acceptor
- Following excitation, the donor passes on the absorbed energy onto the acceptor
  - The donor, thus, does not fluoresce, save for a small amount of background fluorescence
- The acceptor then fluoresces at a higher wavelength/lower frequency
  - Due to energy loss to various factors during transfer

Fluorescence resonance energy transfer (FRET)



Source: http://www-cellbio.med.unc.edu/facilities/fret.htm

Tandem dyes

- In order for FRET to occur, the fluorochromes need to be in close proximity to each other
  - 2-3nm in general, but possible up to 100nm
- This can be achieved on purpose by covalently binding two fluorochromes to each other → tandem dyes
- Remarks:
  - Tandem dyes puts more spectral separation between the excitation frequency and the emission frequency
    - At a slight expense of the “quantum efficiency”
  - The excitation-emission properties of the fluorochromes must be suitable for FRET to occur
  - Tandem dyes tend to be light sensitive
    - Need to be stored away from light
  - Elimination of the spectral overlap from data is more complicated compared to using single fluorochromes

Quantum dots

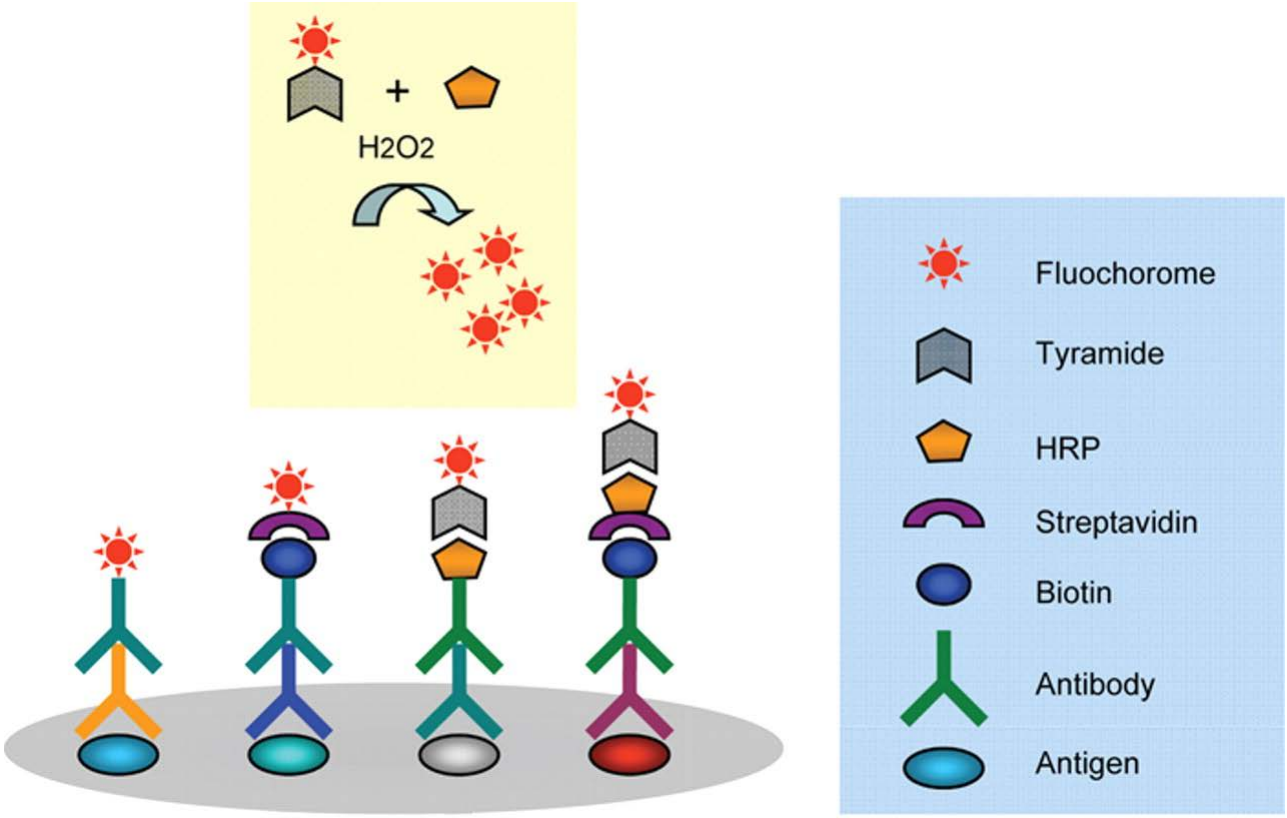
- Quantum dots (Qdot®; Quantum Dot Corp., Hayward, CA) are nanoparticles design specifically for on-demand fluorescent staining
  - The emission spectrum is controlled by the size as well as the core material
- Quantum dots
  - can be excited by light over a wide spectrum range up to emission
  - possess narrow emission spectra
  - are resistance to degradation effects by incident light
- As a result, they allow easier simultaneous analysis of multiple targets
  - Different quantum dots can be excited by the same source
  - They can be distinguished easily due to their distinct emission spectra

Conjugation of fluorochromes

- Fluorescent staining of arbitrary target molecules can be achieved by conjugating fluorochromes to specific antibodies
  - Antibodies specific to the target molecules

    → Monoclonal antibodies
- The down side is loss of quantum efficiency
  - Results in weaker signal
  - Can be alleviated by amplification with additional fluorochromes

Conjugation of fluorochromes



Source: http://jhc.sagepub.com/content/59/4/382/F1.expansion.html

Fluorochrome combinations for multicolor flow cytometry

- Multicolor flow cytometry uses a combination of several fluorescent dyes
  - Each target molecule of interest is stained with a dedicated dye
  - The presence of the fluorochromes are evaluated by the instrument through the measured fluorescence intensities
    - measured by an array of sensors equipped with optical filters
- For best performance, the combination has to be optimized with regard to
  - excitation spectra (dictated by the light source)
  - overlap between the emission spectra
  - the agreement between the emission spectra maxima and the optical filters

Fluorochrome combinations for multicolor flow cytometry

- Combinations suitable for instruments operated by a single laser at 488nm
  - Argon laser, 15-25mW at 488nm
  - 4 optical filters (EPICS XL):
    - FL1, 525 ± 12.5nm
    - FL2, 575 ± 12.5nm
    - FL3, 620 ± 12.5nm
    - FL4, 675 ± 12.5nm
  - The challenge, then, is to find fluorochromes that
    - can be excited optimally at 488nm and
    - possess sufficiently distinct emission spectra across the 4 optical filter wavelengths
  - For instance:
    - Fluorescein is collected by FL1, but leaks to FL2
    - PE is monitored through FL2, but also leaks to FL1 and FL3
  - The artifacts of this spectral overlap must be removed from data before analysis → compensation

Fluorochrome combinations for multicolor flow cytometry

- Combinations suitable for instruments operated by multiple lasers
  - in addition to a laser at 488nm
  - Excitation at multiple wavelengths
    - allows incorporating additional fluorochromes, including the ones that are not optimally excited at 488nm
    - increases the number of markers that can be monitored simultaneously
  - Additional lasers include
    - Helium-neon at 633nm
    - Helium-cadmium at 325nm
    - Krypton ion at 407-415nm
    - Dye-tunable lasers at the range 560-640nm

Fluorochrome combinations for multicolor flow cytometry

- Remarks:
  - It is possible to incorporate additional light sources using mercury or xenon arc lamps
    - older technology
  - Variations over a conventional device configuration are possible
    - Different lasers can hit the flow stream at the same spot or at different spots
      - Time-synchrony in data acquisition required for the latter
    - The detectors are typically arranged so that
      - the first 4 receive the signals originated by the primary laser at 488nm
      - the successive detectors are assigned to the additional lasers in pairs
    - It is always possible to interchange or alter the optical filters determining the spectral characteristics of the fluorescent light collected at each detector
  - Generally, it is not possible to make full use of the theoretical number of distinctly detectable fluorochromes

Remarks

- Additional constraints limit the use of fluorochromes for flow cytometry applications
  - Fluorescence intensity
  - Extent of non-specific binding
  - Sensitivity to pH
  - Susceptibility to photobleaching
  - Light sensitivity
  - Molecular size

Summary

- Specific binding of fluorochromes allow identifying the presence and amount of target molecules of interest in cells
  - When excited by a laser at an appropriate frequency, fluorochromes emit light following a distinct spectral distribution
  - The strength of the detected light is correlated with the amount of target molecule present in the cell
- In addition to fluorochromes with natural affinity to certain substrates, other target molecules can be stained by fluorochromes conjugated to antibodies
- The arrangement of the instrument enforces constraints on the selection of the fluorochromes for multicolor flow cytometry