Fluorescence
Why is fluorescence spectroscopy potentially interesting in terms of application to tissue?

...cancer progression involves a series of chemical changes some of which involve fluorescent compounds.

- protein expression (Trp)
- metabolic activity (NADH/FAD)
- nuclear morphology

- organization
- structural integrity (collagen)
- angiogenesis
Consistent autofluorescence differences have been detected between normal, pre-cancerous and cancerous spectra.
Vibrational Relaxation

Stokes Shift = emitted light is longer wavelength (lower energy) than excitation wavelength

the emission wavelength is red-shifted relative to the absorbed excitation wavelength
Example: Fluorescence Emission

Stokes Shift Origin:
- Internal conversion/Vibrational relaxation
- Local Environment (Solvent effects and excited state reactions)

http://www.advancedaquarist.com/2006/9/aafeature
transitions between electronic states involved in absorption, fluorescence, etc
Quantitative Fluorescence Spectroscopy

\[
S(\lambda_i, \lambda_j) = I(\lambda_i) \left( \frac{\Omega}{4\pi} \right) \sum_{k=1}^{N} 2.3C_k\varepsilon_k(\lambda_i)\phi_k(\lambda_j)
\]

- \( S(\lambda_i, \lambda_j) \) --> Measured fluorescence (intensity) of a dilute solution
  - \( i \) denotes excitation wavelength
  - \( j \) denotes emission wavelength
- \( N \) noninteracting fluorophores (\( k = 1 \) to \( N \))
- Geometric Collection factor: \( (\Omega/4\pi) \)
- Normalized to the excitation intensity, \( I(\lambda_i) \) and the sample thickness, \( L_{dil} \)
- Each fluorophore can be characterized by:
  - a concentration, \( C_k \)
  - a wavelength dependent molar absorption coefficient, \( \varepsilon_k(\lambda_i) \)
  - a wavelength dependent fluorescence quantum yield, \( \phi_k(\lambda_j) \)
What kinds of things in tissue fluoresce*?

- **Endogenous Fluorophores**
  - amino acids
  - structural proteins
  - enzymes and coenzymes
  - vitamins
  - lipids
  - porphyrins

- **Exogenous Fluorophores**
  - Photosensitizers

Generally, things that fluoresce...

  - Aromatic, organic molecules
    - have lots of conjugated double bonds

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Respiratory Enzymes: NADH, FAD, Cytochrome $a_3$

- These enzymes play a key role in providing the proton-motive force necessary for oxidative phosphorylation.
- If tissue is oxygen starved, [NADH] and [FADH$_2$] will be enhanced.
- Reduced NADH conc. is indicative of high oxygen consumption and is characteristic of tumor tissue.

- NADH (FAD) strongly fluoresce while NAD$^+$ (FADH$_2$) does not.
- Cytochrome $a_3$ has a prominent absorption peak at $l = 840$ nm.
A Variety of Ways to Measure Fluorescence

• **UV-Visible (optical) Spectroscopy**
  
  – Modes of Fluorescence spectroscopy (some, not all)
    
    • **Excitation spectrum**
      – Hold emission wavelength fixed, scan excitation wavelength
      – Reports on absorption structure
        » reflects molar absorption coefficient, $\varepsilon_k(\lambda_i)$
    
    • **Emission spectrum**
      – Hold excitation wavelength fixed, scan emission wavelength
        » reflects fluorescence quantum yield, $\phi_k(\lambda_j)$
    
    • **Composite: Excitation-Emission Matrix**

• **Fluorescence lifetime**
  
  – Look at how the excited state depopulates as a function of time
    » Characterizes molecular species
    » Reports on molecular dynamics and environment

Fluorescence Spectroscopy: Challenges

- Relatively broad, featureless spectra can be difficult to interpret (e.g. mixtures of fluorophores, such as tissue)

- Quantitative fluorescence in scattering samples such as tissue
  - Account for effects of scattering and absorption
    - Must develop and apply models of light propagation to fluorescence spectra

- Minimize the likelihood of changes in the sample that result from the measurement process
  - light too intense --> photobleaching
  - sample heating --> fluorescence quenching

- Must choose system components that will not contaminate signal
  - e.g. many types of glass fluoresce, as do adhesives

- Must ensure that excitation light is thoroughly rejected from detection scheme
  - minimize stray light or it may swamp signal of interest
Fluorescence Spectroscopy: Instruments

Excitation and Emission Paths

*Orthogonal to minimize source impact on detector*

Isotropic fluorescence
Optical Filters (interference and absorbing pigment)

- Long/short pass filters transmit wavelengths above/below a cut-on/off wavelength (interference)

- Band pass/notch filters transmit/reject wavelengths in a narrow range around a specified wavelength
  - Bandwidth can be specified

Good for getting rid of laser interference only
Optical Filters (interference and absorbing pigment)

- **Dichroic filters** transmit and reflect wavelengths above/below a wavelength (interference)

- **Neutral Density filter** is a nondiscriminant intensity reducing filter
Fluorescence Spectroscopy: Instruments

Endoscope

Microscope

Surgical field

Excitation Volume

Intensity Control

Light Source (Laser)
Fluorescence Spectroscopy: Indocyananine Green

http://omlc.org/spectra/icg/
Fluorescence-guided Surgery

John Frangioni Laboratory: Beth Israel Deaconess Medical Center

Lymph node identification and characterization: *NIR fluorescence*
Fluorescence-guided Surgery

• Video Link
Fluorescence Spectroscopy: anisotropy

anisotropy: \( r = \frac{I_{\text{par}} - I_{\text{perp}}}{I_{\text{par}} + 2I_{\text{perp}}} \)
Fluorescence Spectroscopy: lifetime

Fluorescence Lifetime Measurements

Time Domain

Frequency Domain

$\tau_\phi = \omega^{-1} \cdot \tan(\Delta\phi)$

$\tau_M = \omega^{-1} \cdot (M^{-2} - 1)^{1/2}$

$M = F_1 E_0 / F_0 E_1$

Figure 1

Intensity vs. Time

Modulated Waves

http://www.olympusmicro.com/primer/techniques/confocal/applications/flimintro.html
Fluorescence Spectroscopy: \textit{lifetime}

MCF10A (Non-malignant, fibrocystic)

Acini Formation-TPEF

NADH fluorescence

day1  day2  day3  day5  day7
day14  day12  day10  10um
Metabolism Changes During Acini Formation

PME

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Fluorescent lifetime

Phasor Plot

![Phasor Plot Image](image7.png)
Measuring Keratinocyte Metabolism

NADH fluorescence of Keratinocytes: Compare near stratum corneum to basal layer

Z = 25-30 µm

Z = 35-40 µm

20 µm
In vivo NADH fluorescence of Keratinocytes near basal layer

Arterial Occlusion

Normalized Mean Intensity (a.u.)

20 µm

depth: 35-40 µm
In vivo NADH fluorescence of Keratinocytes

Arterial Occlusion

Keratinocytes close to the basal layer are regulated by vascular oxygen
In vivo NADH fluorescence of Keratinocytes
Arterial Occlusion

Keratinocytes close to the basal layer are regulated by vascular oxygen.
In vivo NADH fluorescence of Keratinocytes

**Arterial Occlusion**

- **t=7 min**

- **Depth:** 35-40 µm

*Keratinocytes close to the basal layer are regulated by vascular oxygen*
NADH fluorescence of Keratinocytes near stratum corneum

Arterial Occlusion

depth: 20-25 µm

Keratinocyte fluorescence in stratum granulosum unchanged

Metabolism: either atmospheric oxygen or functionally anaerobic
NADH fluorescence of Keratinocytes near stratum corneum
Arterial Occlusion

**ETC under oxygen deprivation conditions:**

\[
NADH \rightarrow NAD^+ + H^+ + 2e^- \rightarrow 2e^- + 2H^+ + 1/2 O_2 \rightarrow H_2O
\]

Keratinocyte fluorescence in stratum granulosum unchanged

Metabolism: *either atmospheric oxygen or functionally anaerobic*