Lecture 9: Spectral Phasors
Is there anything in common between lifetime and spectra?

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Spectral Phasor
Outlines

- Lifetime and spectral phasor: Similarities and differences.
- SP Formalities
- How its work and its properties?
- Instrumentation
- Solvatochromic properties of ACDAN (Methyl)/LAURDAN (Lauryl)
  - Location at membrane and lateral order sensing
  - Generalization Polarization Function (GP)
- Cuvette SP application
  - Effect of cholesterol in Binary membranes
  - Thermotropic transition of a Biological Membrane (Pulmonary Surfactant)
- Image SP application
  - In cellulo hydration and evolution of Lamellar Bodies.
  - Water activity inside the nucleus and its correlation with chromatin compaction
- Conclusions
Lifetime and Spectra
Similarities and differences.

**Lifetime Exponential decay ⇒ Universal Circle**

- Lifetime $\uparrow$, phasor shift
- Angles $\uparrow$
- Modulation $\downarrow$

**Spectral shift ≠ Universal Circle**

- Spectrum $\Rightarrow$ red, phasor shifts
- Angles $\uparrow$
- Broader Spectrum
- Modulation $\downarrow$
Emission spectra is Fourier transformed (cosine sine). A polar plot is constructed using the real (G) and imaginary (S) part of the transform.

Basic mathematical formulas:

\[ G_S = \frac{\int_{\lambda_{\text{min}}}^{\lambda_{\text{max}}} I(\lambda) \cdot \cos \left( \frac{2\pi n(\lambda - \lambda_{\text{min}})}{\lambda_{\text{max}} - \lambda_{\text{min}}} \right)}{\int_{\lambda_{\text{min}}}^{\lambda_{\text{max}}} I(\lambda)} \]

\[ S_S = \frac{\int_{\lambda_{\text{min}}}^{\lambda_{\text{max}}} I(\lambda) \cdot \sin \left( \frac{2\pi n(\lambda - \lambda_{\text{min}})}{\lambda_{\text{max}} - \lambda_{\text{min}}} \right)}{\int_{\lambda_{\text{min}}}^{\lambda_{\text{max}}} I(\lambda)} \]

n = harmonic number

**Spectral Phasor analysis (highlights)**

- Model Free.
- Requires no prior knowledge.
- Combines traditional spectral analysis and phasor analysis.
- Doable in cuvette and in a microscope
- Enables the identification of molecular environment.

- SP position is related to the spectrum center of mass and width.
- Linear property of the phasor approach (follow simple vector math).
- Reciprocal projection: ROI can be selected in the SP plot and the selection can be projected back to the image (basic segmentation).
- Higher harmonic increase the resolution and keep all the phasor properties.
Commercial instruments with spectral resolution:

- **Zeiss LSM 880 (510,710,780)** (simultaneously 32 channels)
- **Leica TCS SP8 X (SP5)** (complete excitation emission spectra)
- **PARISS® Hyperspectral Microscope from LightForm Inc.** (spectrograph coupled to scientific CCD)
- **Homebuilt camera based instruments** (Andor iXon Ultra EMCCD with Shamrock 303i spectrograph)
Spectral phasor plots
How its work and properties

ACDAN in A549
- Weber G, Farris FJ. Biochemistry. 1979

- Dipole moment increase after excitation.

- Spectral shift = Dipolar reorganization around the excited state dipole.
LAURDAN (Lauryl)
Location at membrane and lateral order sensing

Bulk water ($10^{-12}$ sec)

Bound water ($\sim 10^{-9}$ sec)

PRODAN

Cholesterol

$T > T_m$

$L_\alpha$ - liquid disordered

$L_{\beta'}$ - gel phase

$T < T_m$

$L_0$ - liquid ordered

Parasassi T et al 1998
Generalized Polarization function (GP)

Generalized Polarization (GP) = \( \frac{I_B - I_G}{I_B + I_G} \)

GP is additive

Two/three components membrane
DOPC:DPPC (1:1 mol) + ∆%Cholesterol

DOPC:DPPC ⇒ (fluid/gel)
DOPC:DPPC+5% Cholesterol ⇒ (fluid/gel)
DOPC:DPPC+10% Cholesterol ⇒ (fluid/gel/Lo)
DOPC:DPPC+20% Cholesterol ⇒ (fluid/Lo)
DOPC:DPPC+50% Cholesterol ⇒ (Lo)

Native Pulmonary Surfactant is composed by at least 100 different lipids + 4 proteins, we want to characterize the thermotropic transition using LAURDAN fluorescence.

Spectral phasor plots
How about images? Lamellar bodies (LB) organization in vivo?

Mature LB
Secreted LB
Composite bodies (CB; immature LBs)
Multivesicular body (MVB)

Pneumocyte type II cell

Biophysical Journal
Volume 109, Issue 11, 1 December 2015, Pages 2295–2306

Article
Pneumocytes Assemble Lung Surfactant as Highly Packed/Dehydrated States with Optimal Surface Activity
Alejandro Cerrada¹, Thomas Hallier², Antonio Cruz³, Jesús Pérez-Gei¹

• Watanabe Y. et el, Ped.Research (2013)
• Dr. Donald Fawcett, M. Williams. http://www.visualsunlimited.com/image/I0000RApWbJL.cBw
• Stahlman MT. Lab Invest 2000
• OCHS M. THE ANATOMICAL RECORD PART A (2004)
Spectral phasor plots
How about images? The Lamellar bodies maturation in A549 cells.

- Intracellular LB-like structures grow first in size, followed by sharp dehydration.

• Secreted LB-like structures are more hydrated than the intracellular ones.
• Non-lamellar phases can play a role in intracellular LB-like structures.

Spectral phasor plots
How about images? The Lamellar bodies maturation in A549 cells.
Spectral phasor plots
Exploiting the linear combination. LAURDAN vs Nile red cell membrane profile in A549

Correlation of LAURDAN and Nile Red Fluorescence in LB of A549 cells.
Identity of LBs:
Co-localization of ABCA3 transporter-GFP with the Nile Red fluorescence in the LB of A549 cell.
Spectral phasor plots
What about water activity inside the interphase nucleus?

Chromatin organization and diffusion dynamics inside the nucleus

- What is the state of water inside the nucleus?
- What is the relationship between water activity and the chromatin state?

- EGFP diffusion by pCF
- DNA densities (high/low)
- Connection between high/low DNA densities can happen in longer time scale (300ns).

https://www.pinterest.com/pin/338403359474084683/
Hinde E. et al. PNAS (2010)
Spectral phasor plots
Exploiting the linear combination. Dipolar relaxation inside NIH3t3 nucleus using ACDAN

-Nucleus has the most dipolar relaxable water compared with the whole cell.

-Inside the nucleus, it is possible to identify a gradient of dipolar relaxation by the ACDAN spectral shift associated with at least two different environments.

Malacrida et al-2016 in preparation
Spectral phasor plots
Exploiting the linear combination. Dipolar relaxation vs Chromatin markers

ACDAN+NucRed, $\lambda_{ex} 900$nm

ACDAN+H2B_dsRed, $\lambda_{ex} 900$nm

-Eu and heterochromatin display different dipolar relaxation.
- Euchromatin $\uparrow$ water activity
- Heterochromatin $\downarrow$ water activity

Malacrida et al-2016 in preparation
Spectral phasor plots
What are the advantages of spectral phasor approach vs ratiometric?

• It is possible to associate molecular species as phasor plot fingerprint without performing any fitting or modelling.

• We can demix the contribution of multiple fluorescent species in a blind way (non-previous knowledge).

• The combination of fluorescent species in the same pixel follows a simple vector linear rule.

• Straightforward quantitative approach.

• Characterization in a model-free fashion of lateral segregation in membranes.

• Exploiting SP fingerprints for lamellar membranes we can detect the occurrence of non-Lamellar phases in vivo.

• First evaluation of the water activity inside the cell and new insights in the chromatin macromolecular signature.
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