Imaging Hydroxyapatite in sub-RPE Deposits by Fluorescence Lifetime Imaging Microscopy (FLIM)

HOT TOPIC

#A0020

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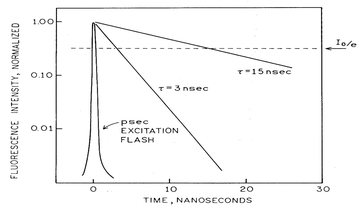
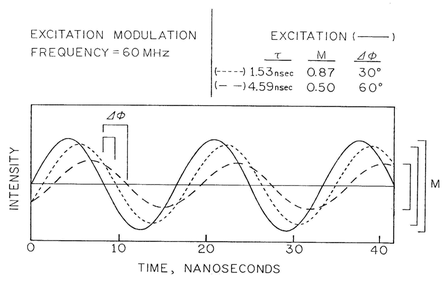
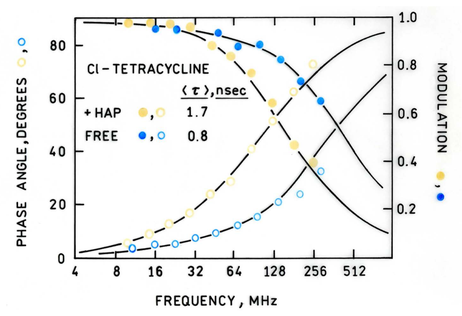
Purpose: Recently, we found that small spherules (~2 um) of hydroxyapatite (HAP) were present in sub-RPE deposits and have obtained evidence that they may nucleate the growth of the deposits (PMID: 25605911). The HAP spherules can be imaged with fluorescent stains such as LiCor Bone Tag or OsteoSense in vitro, but their use in vivo might be difficult especially as their safety in humans is unproven. Tetracycline antibiotics are well known to bind to HAP with a substantial concomitant increase in fluorescence, and their safety in humans and animal models is well established. Here we examined whether tetracyclines might be suitable to image the HAP spherules by fluorescence lifetime imaging microscopy (FLIM) to overcome challenges with detection due to the autofluorescence of the RPE.



Principle of Fluorescence Lifetime Imaging Microscopy (FLIM): Fluorescence lifetime imaging differs from regular fluorescence imaging in that the contrast in the image arises not from differences in intensity or wavelength, but differences in the fluorescence decay time or lifetime. In the time domain (TD), a fluorophore ensemble excited with a brief (psec) flash of light exhibits a log-linear decay , where the time the intensity takes to fall to 1/e of its initial values is the lifetime, τ (see **Figure above**). In the frequency domain (FD), the fluorophores are excited with modulated light at some frequency ω; the fluorescence emission is phase-delayed by an angle ϕ and demodulated by a factor m with respect to the excitation (see **Figure upper right**). The lifetime in FD measurements of a single class of emitters are simple functions of ω and ϕ or m. When multiple lifetimes are present in TD, the time-dependent intensity data may be fit to a decay model and the accuracy judged by goodness-of-fit; in FD, ϕ and m are measured at multiple frequencies and the lifetimes and preexponential factors α of the components obtained by a comparable fitting procedure.



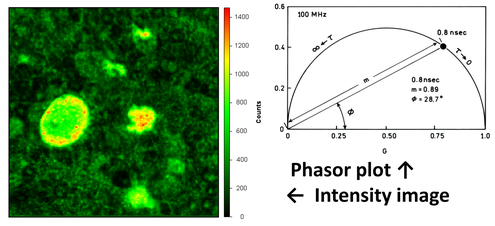


Lifetimes of Cl-Tetracycline in solution and bound to HAP We measured phase angles ϕ (open circles) and modulations m (filled circles) at frequencies from 10-300 MHz for Cl-Tet free in buffer (yellow circles) and bound to hydroxyapatite (blue circles). The lines indicate the best two component fits to the data; the derived average lifetimes <τ> were 0.8 and 1.7 nsec for free and bound, respectively.

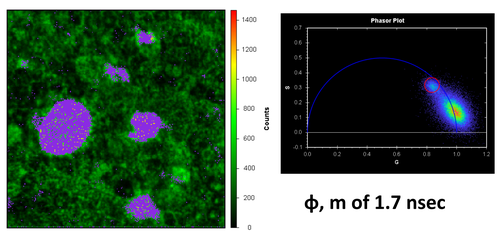
Phasor Plots of ϕ and m (right →): We can highlight pixels in the images whose ϕ's and m's fall in a particular range, which reflects their lifetime properties. The phasor plot maps the position of each pixel as x = m cosϕ and y = m sinϕ at some frequency ω. Thus pixels with x = 0.82 and y = 0.31 correspond to ≈ 1.7 nsec and x = 0.95 and y = 0.12 correspond to ≈ 0.6 nsec.

FD FLIM Images of Human Retina Stained w/Cl-Tetracycline: We stained flat mounts of fixed retina preparations from a 94-year old female donor with Chlortetracycline (Amresco) and imaged them by FLIM in an ISS Alba confocal fluorescence lifetime microscope with 473 nm excitation and 520 nm emission. Our goal in this preliminary study was to observe the differences in contrast between HAP in the drusen and the tissue background using 3 different FD FLIM display techniques: 1) lifetime images using average lifetimes, 2) images of the phase angles of individual pixels at a selected frequency, and 3) plots of pixels having preselected phase and modulation values corresponding to the lifetime of Cl-Tet bound to HAP (phasor plot).

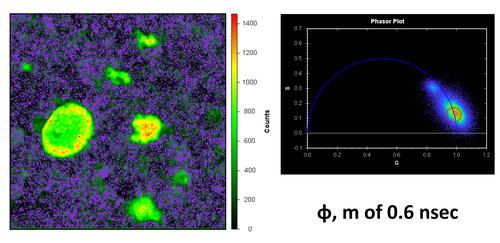
Mapping Pixels with Selected Phase and Modulation (Phasor Plot)



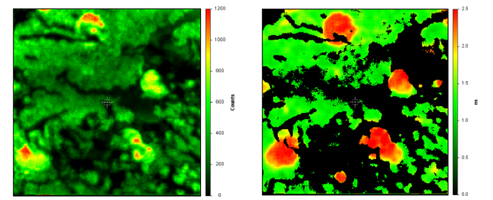
Phasor plot ↑
← Intensity image



ϕ, m of 1.7 nsec

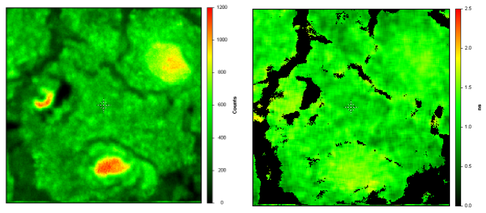


ϕ, m of 0.6 nsec



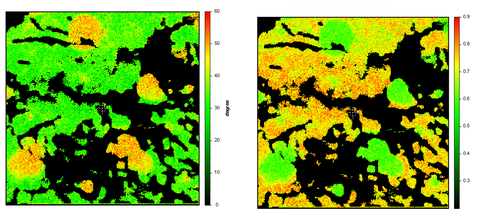
Images of Average Lifetimes (Stained)

Left panel is fluorescence intensity image of drusen, right panel pixel values color-coded with average lifetime. Bright areas fit τ_1 = 1.91 nsec, α_1 = 0.95; τ_2 = 11.5 nsec, α_2 = 0.05.



Lifetime Images of Unstained Specimens

Lifetime image shows low contrast of drusen with background. Bright areas fit τ_1 = 0.89 nsec, α_1 = 0.87; τ_2 = 4.9 nsec, α_2 = 0.13.



Images of Phases and Modulations (80 MHz)

Images are color-coded of phase angle ϕ (left) and modulation m (right) of same field as top row; note for HAP-bound Cl-Tet, ϕ ≈ 40 degrees and m ≈ 0.67 at 80 MHz

Conclusions: Based on these results it seems feasible to image tetracycline-stained HAP spherules in the retina, and based on the pioneering in vivo measurements of the Schweitzer (PMID 22511622) and Zinkernagel (*IOVS* **55**:2106–2113(2014)) groups it may be feasible to use this in vivo following staining with an orally administered tetracycline

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