Infusion labeling and FLIM imaging of hydroxyapatite spherules in human sub-RPE deposits

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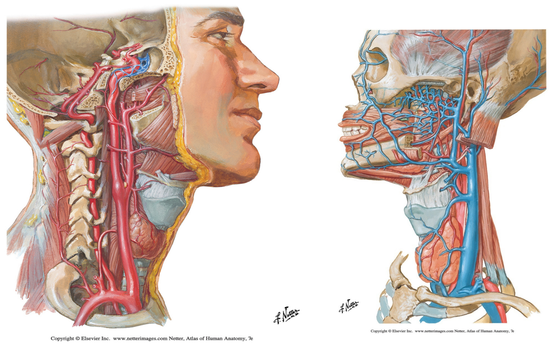
A0309

PURPOSE: Recently, we found that hydroxyapatite (HAP, $Ca_5(PO_4)_3OH$), the hard mineral form of calcium phosphate in bone and teeth, is a principal constituent both of microscopic spherules, precursors for drusen formation (PMID 25605911), and nodules, a progression marker for geographic atrophy (PMID 30404862) in AMD. We proposed that HAP deposition may precede the development of or be associated with progression to AMD, and thus HAP could serve as a robust biomarker for AMD screening. HAP can be labeled and imaged by microscopy with fluorescent probes designed for bone growth studies (LiCor BoneTag and Perkin Elmer OsteoSense) and compounds such as the tetracyclines. One challenge is administering such stains to humans in a screening scenario. While Bone Tag and OsteoSense stains perform well *in vivo* in animal models, they are not yet approved for human use. By comparison, humans have been treated with oral tetracyclines for decades, and their pharmacokinetics are known. Thus we sought to stain HAP deposits *in situ* by infusion through the vasculature.

OBJECTIVE: To test the efficacy of staining retinal drusen by infusion through the vasculature using chlortetracycline, and imaging flat mounted retinas using FLIM

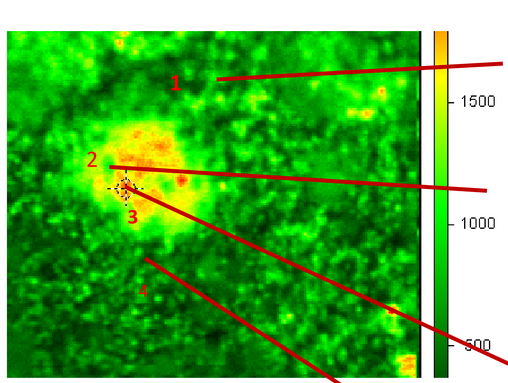
METHODS: All experiments were performed with oversight and approval from the UMB IRB. For labeling we perfused donor cadavers (post mortem time 6-12 hours, >55 years old) with chlortetracycline (Cl-Tet) in PBS for one hour at therapeutic levels by infusion through the left interior carotid artery, followed by a flush with PBS. Entry of Cl-Tet was observed in scleral blood vessels visually. Eyes were enucleated, the RPE-choroid complex flat mounted on slides, and sub-RPE deposits imaged by brightfield and fluorescence lifetime imaging microscopy (FLIM) as described (Szmacinski, et al., *Proc. SPIE* 10484 (2018)).

BACKGROUND : Early detection of drusen before irreversible vision loss has occurred due to age-related macular degeneration (AMD) remains an important goal, particularly as GWAS and other approaches have identified potential targets for therapeutics. We had proposed the imaging of hydroxyapatite (HAP) spherules may serve in this regard, but the existing fluorescent bone stain families are not approved for use in humans, and may be challenging to administer. We found that certain of the tetracycline antibiotics exhibit a substantial increase in fluorescence lifetime upon HAP binding, and the pioneering work of Schweizer, Zinkernagel, and their colleagues showed the background lifetime of the healthy adult retina in this spectral regime is very short. The tetracyclines have been used in humans for decades and their safety and pharmacokinetics are well known; moreover, most are orally bioavailable, which is attractive for screening. Thus we sought to stain the retinas of recently deceased elderly donors by infusion through the vasculature and image sub-RPE deposits by FLIM.

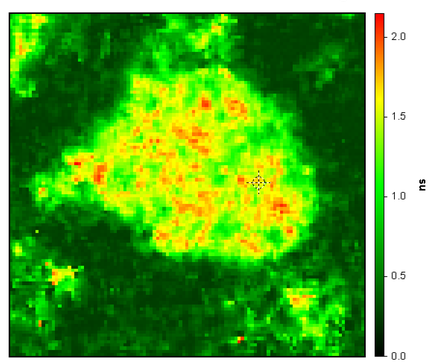


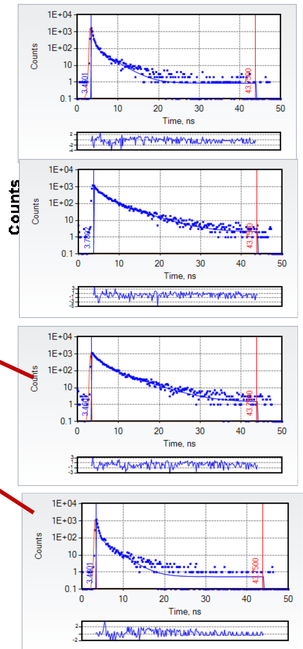
Infusion was performed by exposing the left internal carotid artery and infusing chlortetracycline, with fluid exiting the right jugular vein.

The authors gratefully acknowledge: the Maryland State Anatomy Board and its Directors Robert Wilk and Juan Ortega and staff for their outstanding cooperation and support, Prof. J.R. Lakowicz of the Center for Fluorescence Spectroscopy Core Facility for the use of FLIM, and the following for their financial support: the Bright Focus Foundation (R.B.T., I.L.); the Bill Brown Charitable Trust, the Mercer fund from Fight for Sight, and the Eye Charity of Moorfields Eye Hospital (I.L.) and an unrestricted grant from OPTOS Plc. (I.L.).

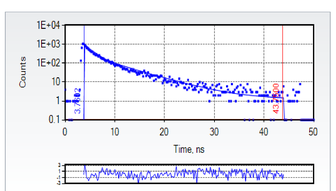


ABOVE: Fluorescence intensity micrograph of flat-mounted left retina of 82 year old female (cause of death: congestive heart failure) following infusion with one liter of 500 mg/L Cl-Tet in PBS and a one hour rinse with PBS. Excitation ≈ 442 nm (150 psec pulse width), emission 50 nm FWHM centered at 520nm. Approximate size of the druse is 50 um. Time domain fluorescence lifetime imaging microscopy (TD-FLIM) data were collected on an ISS ALBA FLIM through a 20x 0.4 objective, and those of indicated points in the image fit to two components (time decays to right) with the fitted parameters summarized in the table to the right.





LEFT: Closeup preexponential-weighted average lifetime $<\tau_\alpha>$ image of drusen above; for the whole image fit to two components yielded $\tau1 = 1.474$ ns , $\alpha1 = 0.99$, $\tau2 = 5.434$, $\alpha2 = 0.01$, $\chi2 = 1.06$. Aggregate decay curve illustrated below:



2-Component Fits to Cl-Tet Infusion-Stained Retina

Field	τ_1	α_1	τ_2	α_2	$<\tau_\alpha>$	χ^2
1	0.36	0.97	2.88	0.03	0.44	1.01
2	1.35	0.93	5.28	0.07	1.62	1.10
3	1.49	0.95	5.81	0.05	1.72	1.31
4	0.34	0.97	2.66	0.03	0.41	0.77

DISCUSSION: Our results to date show that drusen can be stained by infusion with chlortetracycline and imaged by FLIM in the enucleated eye, suggesting that the tetracycline stain may be administered orally. They also suggest that they can be imaged *in vivo* by the FLIO of Schweizer and Zinkernagel owing to the short lifetime of the background fluorescence they measured in the normal healthy retina (Dysli, et al., *Prog. Ret. Eye Res.* 2017). Unfortunately, the slow absorption and excretion of Cl-Tet in vivo (Agwuh and MacGowan, 2006) is infeasible to duplicate in an unpreserved cadaver at room temperature. The safety, oral bioavailability, and clinical experience with the tetracyclines as a class are all very attractive advantages for clinical application.

Remaining issues include determination of how useful a predictive biomarker HAP is for AMD, what optical resolution and sensitivity are necessary for early detection, and which tetracycline(s) are best for this application. Fortunately, aging macaques develop HAP-containing drusen similar to those in humans with AMD, suggesting they will be a useful model in these studies going forward.