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Multiphoton coherence domain molecular imaging with pump–probe optical coherence microscopy

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Abstract

We have developed a high-resolution molecular imaging technique, pump–probe optical coherence microscopy (PPOCM), based on the fusion of pump–probe spectroscopy and optical coherence microscopy. We have demonstrated the prototype system on a fixed human skin sample containing a nodular melanoma. The results indicate that the PPOCM can clearly provide a strong contrast between the melanotic and amelanotic regions. Potential applications of the PPOCM imaging of melanin include the early diagnosis of melanoma and the mapping of tumor margins during excision. The technique may in general be applied to any biological chromophore with a known absorption spectrum.

High-resolution optical molecular imaging currently plays an essential role as a research tool for biology, biochemistry, and the biomedical sciences. The prevailing high-resolution optical molecular imaging modalities based on fluorescence need exogenous tags in order to probe the majority of biomolecular species because of their poor intrinsic fluorescence. In addition to the increased experimental complexity, these tags may potentially interfere with the process under study or even prove toxic to the sample. The need for exogenous tags also impedes applications in humans since any tag must garner FDA approval. It is therefore desirable to develop high-resolution molecular imaging techniques that do not rely on fluorescent tags but rather exploit physical phenomena which are more common in biomolecules [1,2].

Pump–probe spectroscopy is a well-established tool in molecular physics for measuring the spectrum and dynamics of molecular species that are poor fluorophores. The foundation of the technique lies in the detection of transient changes in the probe attenuation induced by the pump radiation. While there are a number of different schemes involving molecular excited states, some of which we have enumerated earlier [3], we are primarily interested in the transient bleaching of the ground state induced and detected by degenerate or near degenerate pump and probe wavelengths. In this manifestation of pump–probe spectroscopy the pump drives molecules to the excited state thereby depleting the ground state population. The intensity of the probe radiation is then stronger when the pump is on as compared to when the pump is off due to the reduced probability of absorption and the increased probability of stimulated emission.

We have recently adapted degenerate pump–probe spectroscopy to optical coherence tomography (OCT) to yield ground state recovery pump–probe optical coherence tomography (gsrPPOCT) [4]. We were able to image a number of endogenous chromophores including hemoglobin in the efferent filament arteries of an adult zebrafish. We were also able to measure and use the ground state recovery time of hemoglobin and Rhodamine 6G as means to separate

signal contributions from both in a mixture. The spatial resolutions in the gsrPPOCT and OCT, 5–10 μm axial and 10–20 μm lateral, are well suited to imaging the micrometer-scale morphology of living tissues.

The work described here represents our effort to extend the resolution of this technique beyond the micrometer-scale morphology to the cellular/subcellular level. To realize that goal we have combined pump–probe spectroscopy with optical coherence microscopy (OCM) to yield pump–probe optical coherence microscopy (PPOCM). OCM is the fusion of low coherence interferometry with confocal microscopy. In the OCM, the scattered light is filtered by both the coherence and confocal gates to yield scattering based images at depths superior to standard confocal microscopy [5]. The addition of pump–probe spectroscopy will introduce a molecular dependent gate, which will enable the simultaneous acquisition of a molecular image and a scattering based image.

Image formation in PPOCM follows from the three gates enumerated above. The coherence and confocal gates combine to form the point spread function (PSF) of a typical OCM system. The coherence gate contributes only in the axial dimension (z) and is given by $\text{PSF}_L(z) = \exp(-z^2 4 \ln(\Delta z)^{-2})$, where Δz is the FWHM of the PSF and we have assumed a Gaussian source spectrum. Note that Δz is the coherence length of the source, which is related to the source center wavelength and bandwidth by $\Delta z = 2 \ln 2 \lambda_0^2 (\pi \Delta \lambda)^{-1}$, where λ_0 is the center wavelength and $\Delta \lambda$ is the source FWHM. The confocal gate contributes in both the axial and radial dimensions. The confocal PSF, following Sheppard and Gu [6] is given by $\text{PSF}_C(r, z) = I(r, z)^2$, where $I(r, z)$ is the intensity in the focal region. The PSF for OCM is then the product of the two,

$$\text{PSF}_{\text{OCM}}(r, z) = \exp\left(-\frac{z^2 4 \ln 2}{(\Delta z)^2}\right) I(r, z)^2. \quad (1)$$

In the two limiting cases, long coherence length or long depth of focus, Eq. (1) becomes the PSF for a confocal microscope or an OCT system, respectively.

The molecular gate in the PPOCM is due to the overlap of the pump and probe beams. Following Dong *et al.* [7], in the absence of a confocal gate the PSF due to the overlap of the pump and probe is given by $\text{PSF}_{P-P} = I(r, z) I'(r', z')$, where the prime indicates the pump beam and the transition of the chromophores is assumed to be far from saturation. When combined with the confocal gate the PSF is improved to $\text{PSF}_{P-P} = I(r, z) I'(r', z') I(r, z)$. The addition of the coherence gate leads us to an equation for the PSF in PPOCM,

$$\text{PSF}_{\text{PPOCM}}(r, z) = \exp\left(-\frac{z^2 4 \ln 2}{(\Delta z)^2}\right) I(r, z)^2 I'(r', z'). \quad (2)$$

Comparing Eqs. (1) and (2) it is clear that the spatial resolution of the PPOCM may exceed that of the base OCM system depending on the focusing of the pump beam. In the limit that the pump beam is unfocused, $\text{PSF}_{\text{PPOCM}}$ is equivalent to PSF_{OCM} . Similarly as we approach saturation of the transition, $\text{PSF}_{\text{PPOCM}}$ will approach PSF_{OCM} . Even if the transition is completely saturated by the pump along the entire beam path the spatial resolution will never deteriorate below that of the base OCM system. This is important, since the maximum PPOCM signal is achieved when the transition is saturated. If our technique relied solely on the overlap (PSF_{P-P}), the maximum signal would be accompanied by a drastic deterioration of the spatial resolution.

A probe bandwidth larger than the bandwidth of the transition being probed will not improve the PPOCM resolution, because the additional bandwidth will not be modulated by the pump. Additionally, subresolving the confocal gate by decreasing the source coherence length leads to an ambiguity in the spatial position where the absorption occurs, because absorption is an integrative process in the path length. Recovering the depth dependent absorption would require the computation of the derivative image [4].

We have selected melanin as the initial target chromophore for our PPOCM system. Melanin is one of the most abundant chromophores in the human body. The different forms of melanin play roles in the functions of the brain, ear, eyes, and skin. There are a number of potential applications for the imaging of melanin. One of the most important is as a tool to investigate and potentially diagnose melanoma, a particularly aggressive cancer starting in the melanocytes and the most common form of skin cancer.

The optical system is shown schematically in Fig. 1. A tunable bimodal source was generated by launching approximately 250 mW of light from a tunable (680–1080 nm) 140 fs Ti:sapphire laser (80 MHz) into a 6.5 cm length of the polarization-maintaining high-NA single-mode fiber. The center of the spectrally broadened pulse [Fig. 2(A)] was removed by filtering with a polarizer to yield the bimodal spectrum shown in Fig. 2(B). The probe light ($\Delta z \sim 50 \mu\text{m}$) was launched into an interferometer based on a Mach–Zehnder design. A piezoelectric transducer (PZT) in the reference arm, driven at resonance (57.25 kHz) by a sine wave, provided a carrier frequency for the interferometric signal. The chopped (3.4 kHz) pump beam passed through an optical delay line, which also rotated the polarization by 90° . The pump and probe, combined in a polarizing beam splitter, were focused onto the sample using a 0.8 NA water immersion objective which provided a sub- $0.5\text{-}\mu\text{m}$ lateral resolution and a measured $\sim 2 \mu\text{m}$ axial (confocal gate) resolution. A 2×2 (50/50) single-mode fiber coupler served as the confocal pinhole as well as the final beam splitter in the interferometer. The two outputs of the fiber coupler were connected to a 125 kHz dual balanced detector. *En-face* imaging was accomplished by translating the sample using a high precision stage. The measured signal-to-noise ratio of the OCM system was 122 dB, 5 dB from the shot-noise limit of 127 dB.

The interferometric signal (S_{OCM}) was extracted as in [8] by taking the square root of the sum of the power at the PZT fundamental modulation frequency (P_1) and the second harmonic (P_2), $S_{\text{OCM}} = \sqrt{P_1 + P_2}$. As long as the amplitude of the PZT motion was equal to 0.84 of a fringe then S_{OCM} as calculated above was independent of the phase of the interference signal. The amplitude modulation of the pump beam introduced sidebands on P_1 and P_2 at $\pm$ the pump modulation frequency when there was a pump induced change in the probe signal amplitude. The sidebands, corresponding to the PPOCM signal, were processed similar to the OCM signal, $S_{\text{PPOCM}} = \sqrt{P_{1+} + P_{2-}} + \sqrt{P_{1-} + P_{2+}}$, where the + and – subscripts indicate the plus and minus sidebands, respectively.

The sample, shown in Fig. 3(A), was prepared by cutting a ~ 1 mm thick section from fixed human skin containing a nodular melanoma perpendicular to the tissue surface. A region of tissue in the boundary area between melanotic (left) and amelanotic (right) regions, indicated in Fig. 3(A), was scanned with the PPOCM system over a field of view of $135 \mu\text{m} \times 90 \mu\text{m}$ at a depth of $\sim 26 \mu\text{m}$. The pump and probe powers on the sample were 2.16 mW and 1.17 mW, respectively, with the pump leading the probe by 50.5 ps.

The resulting OCM and PPOCM images acquired simultaneously are shown in Figs. 3(B) and 3(C), respectively. The melanotic regions should have stronger attenuation due to absorption; however, in general they are also more highly scattering than the amelanotic regions because of the melanin granules. The net result is a fairly poor contrast between the melanotic and amelanotic regions in the OCM image with the melanotic regions appearing brighter. The

PPOCM image shows no signal in the amelanotic regions and a strong signal in the melanotic regions, thus providing a strong contrast. While the melanin is largely isolated on the left side, the PPOCM image clearly indicates small “islands” of melanin deposits in the otherwise amelanotic region on the right. These islands are not discernable in the OCM image; however, they are consistent with the light microscope image in Fig. 3(A), which indeed shows small black areas infiltrating the amelanotic region on the surface of the sample. The PPOCM image shows a strong molecular contrast and enables the identification of molecular deposits not clearly seen in the OCM image.

In addition to just providing molecular contrast, we would like to map the relative concentration of the target chromophore. Unfortunately, while the PPOCM signal is a function of the chromophore concentration (C) it is also a function of the tissue reflectivity (R), pump power (P_{pu}), and probe power (P_{pr}). The PPOCM image is therefore a poor approximation of the relative chromophore concentration. At a fixed depth, P_{pu} and P_{pr} reaching that depth is approximately constant over small lateral distances; hence the most important variable is R . We can take advantage of the fact that we also effectively measure the sample reflectivity simultaneously in the OCM image. The OCM image is formally a function of P_{pr} and R ; hence the ratio PPOCM/OCM is a function of C and P_{pu} and is a reasonable approximation of the relative chromophore concentration at a given depth in the tissue. This ratio is shown in Fig. 3(D). Before taking the ratio, both the OCM and PPOCM images were normalized and spatially averaged over 3 pixels ($1.5 \mu\text{m}$) in both dimensions to reduce the noise. The PPOCM signal was additionally thresholded at 7% of the maximum intensity. While the spatial averaging was insufficient to remove the speckle pattern from the OCM and PPOCM images, it is suppressed in the ratio image, because the speckle is perfectly correlated in the OCM and PPOCM images. The PPOCM/OCM ratio image provides the relative chromophore concentration over small lateral distances and cancels the speckle noise to yield a high-contrast low-noise molecular image.

In conclusion, we have developed a PPOCM system and used it to image melanin in human skin tissue with a nodular melanoma. The results indicate that PPOCM can clearly provide a strong contrast between the melanotic and amelanotic regions, which is not available in the scattering based OCM image. Potential applications of the PPOCM imaging of melanin include the early diagnosis of melanoma and the mapping of tumor margins during excision. While we have demonstrated the prototype PPOCM system on melanin the technique is more general and may be applied to any biological chromophore with a known absorption spectrum and sufficient concentration.

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Fig. 1. Schematic diagram of the pump-probe optical coherence microscope: PMF, high-NA polarization-maintaining single-mode fiber; Obj, objective; BS, beam splitter; PBS, polarized beam splitter; LPF, long pass filter; SPF, short pass filter; DM, dichroic mirror; 2×2, 2×2 50/50 fiber coupler.

Fig. 2. (A) Representative source spectrum after exiting the PMF. (B) Representative source spectrum after the polarizer. The short wavelength band was used as the pump, and the long wavelength band was used as the probe.

Fig. 3. (Color online) (A) Light microscope image of the fixed nodular melanoma sample. The scale bar is $100\ \mu\text{m}$. The box indicates the approximate region where the OCM and PPOCM images were recorded. (B) OCM image. The scale bar is $20\ \mu\text{m}$. (C) PPOCM image. (D) PPOCM/OCM ratio image depicting the relative melanin concentration. Images (B)–(D) have the same field of view and scale.