Optical metabolic imaging of live tissue cultures

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ABSTRACT

The fluorescence properties, both intensity and fluorescence lifetime, of NADH and FAD, two coenzymes of metabolism, are sensitive, high resolution measures of cellular metabolism. However, often in vivo measurements of tissue are not feasible. In this study, we investigate the stability over time of two-photon auto-fluorescence imaging of NADH and FAD in live-cultured tissues. Our results demonstrate that cultured tissues remain viable for at least several days post excision. Furthermore, the optical redox ratio, NADH fluorescence lifetime, and FAD fluorescence lifetime do not significantly change in the cultured tissues over time. With these findings, we demonstrate the potential of sustained tissue culture techniques for optical metabolic imaging.

Keywords: multi-photon fluorescence, cellular metabolism, clinical translation, cancer,

1. INTRODUCTION

Optical metabolic imaging (OMI) is uniquely sensitive to metabolic changes due to tumorigenic transformation1-3. OMI utilizes the fluorescence properties of NADH and FAD, two co-enzymes of metabolic reactions. OMI endpoints include the optical redox ratio, the fluorescence intensity of NADH divided by the fluorescence intensity of FAD, the NADH fluorescence lifetime, and the FAD fluorescence lifetime. The optical redox ratio provides a dynamic readout of cellular metabolism, with increased redox ratio (NADH/FAD) often observed in malignant cells exhibiting the Warburg effect (increased glycolysis even in the presence of oxygen)5. The fluorescence lifetime values report differences in the conformation and binding of the fluorophore, as well as microenvironment changes, such as pH, temperature, and proximity to quenchers such as free oxygen6.

Development of a live-culture tissue technique combined with OMI would enable OMI measurements from excised tissues in the place of in vivo measurements. Often in vivo measurements are not feasible due to disease site and patient/clinician cooperation. Here we investigate two tissue culture techniques. In the first, bulk tissue samples (~6mm x 1mm) are placed in chilled tissue media and OMI performed every 4 hours up to 24 hours. In the second, tumor cells are grown in vitro in organoid (or macro-suspensions of 100-300 μm diameter) cultures which retain all the innate components of the tumor.

2. METHODS

2.1 Live-tissue culture and organoid generation protocol

This study was approved by the Vanderbilt University Animal Care and Use Committee and meets the National Institutes of Health guidelines for animal research. First, we determined the length of time OMI endpoints remained stable in bulk, freshly excised tissue maintained in chilled media. For this experiment, we used in vivo and freshly excised tissue from the hamster cheek pouch (n=6). For the in vivo imaging, each hamster was anesthetized and arranged on the microscope. Three different locations of the hamster cheek epithelium were imaged at a depth of the spinosum layer for each hamster. After imaging, a biopsy of the cheek pouch...
corresponding to the imaging locations was obtained and the hamster humanly euthanized. The biopsy was immediately placed in chilled media and subsequently imaged over time for the next 24 hours.

For the organoid experiments, organoids were generated from a MDA-MB-361 (human breast cancer cell line) xenograft. The xenograft was grown in the mammary fat of a J:Nu homozygous female mouse (Jackson Labs) by injection of $10^6$ cells in 100 μl of Matrigel. Once the tumor reached ~200 mm$^3$ in volume, the mouse was humanly euthanized, and the tumor harvested. The tumor was processed into macro-suspensions ranging in size from 100-300 μm by mechanical dissociation. The organoids were diluted in primary mammary epithelium cell culture media (PMEC) and Matrigel (1:2, media:Matrigel). These cultures were placed on glass bottom imaging dishes (MatTek Corp), solidified at room temperature, and overlain with PMEC. Organoids were incubated at 37°C with humidity and CO$_2$ maintained for 72 hours. OMI of the organoids (n=6) from was performed at 24, 48, and 72 hours.

2.2 Optical metabolic imaging

OMI was performed on a multi-photon fluorescence microscope customized for time-correlated single photon counting (Prairie Technologies). A 40X water-immersion objective (1.15 NA) or a 40X oil-immersion objective (1.3 NA) coupled the excitation and emission light through an inverted microscope (TiE, Nikon). The excitation light was provided by a titanium:sapphire laser (Chameleon, Coherent), tuned to 750 nm for NADH fluorescence and tuned to 890 nm for FAD fluorescence. Laser power at the sample was 7.5-7.8 mW for NADH fluorescence and 8.4-8.6 mW for FAD fluorescence. Emitted photons were detected by a GaAsP PMT (H7422P-40, Hamamatsu). Customized filter sets isolated NADH emission between 400-480 nm and FAD emission between 500-600 nm.

Fluorescence lifetime images were acquired by time-correlated single photon counting (TCSPC) electronics (SPC-150, Becker and Hickl). Each fluorescence lifetime image (256x256 pixels) was acquired using an integration time of 60 seconds. Photon count rates were maintained above 5x10$^6$ photons per second, and no change in the photon count rate was observed over the course of the image acquisition, ensuring that photobleaching did not occur. The instrument response function (measured from the second harmonic generated signal of urea crystals excited at 900 nm) full width at half maximum was measured to be 260 ps. TCSPC measures the time between a laser pulse and the fluorescence event with a fast detector PMT. A histogram of photon counts (Fig. 1) at each time bin was constructed for each pixel and fit to a two-component exponential decay curve (SPCImage, Becker and Hickl) according to the equation (1) where $I(t)$ is the fluorescence intensity at time $t$ after the laser excitation pulse, $\alpha_1$ and $\alpha_2$ are the fractional contributions of the short and long lifetime components, respectively (i.e. $\alpha_1 + \alpha_2 = 1$), $\tau_1$ and $\tau_2$ are the fluorescence lifetimes of the short and long lifetime components, and $C$ accounts for background light. Mean fluorescence lifetime ($\tau_m$) values for NADH and FAD were computed from the weighted average of the short and long lifetime components (Equation 2).

$$I(t) = \alpha_1 \exp^{-t/\tau_1} + \alpha_2 \exp^{-t/\tau_2} + C$$ \hspace{1cm} (1)

$$\tau_m = \alpha_1 \tau_1 + \alpha_2 \tau_2$$ \hspace{1cm} (2)

The single-component fluorescence lifetime of a fluorescent bead (Polysciences Inc.) verified instrumentation and analysis techniques. The measured fluorescence lifetime of the bead was 2.1 ± 0.1 ns (n = 3), which is consistent with published studies.$^{2,3,7}$
Figure 1: Representative fluorescence lifetime decay curve (red), system response (green), and data points (blue).

Rank sum tests of mean were used to compare mean redox ratio, NADH $\tau_m$, and FAD $\tau_m$, values between in vivo versus live-culture tissue and control IgG versus treatment. A p-value less than 0.05 indicated significance.

3. RESULTS

3.1 OMI of bulk live-cultured tissues

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<thead>
<tr>
<th>In Vivo</th>
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<td>Spinousm</td>
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<td>Redox Ratio</td>
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<td>NADH $\tau_m$</td>
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<td>FAD $\tau_m$</td>
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Figure 2. Representative OMI images of the hamster cheek epithelium in vivo, and from intact tissue cultured for 4 and 12 hours. Each image is 100 $\mu$m x 100 $\mu$m.
Figure 3. Normalized measurements (to the \textit{in vivo} value) of the optical redox ratio (A), NADH mean fluorescence lifetime (B) and FAD mean fluorescence lifetime (C) of live-cultured hamster cheek epithelium tissue over time (n=6)

* \(p<0.05\), ** \(p<0.001\).

Representative OMI images are shown in Figure 2. OMI resolution is adequately high to image individual cells and identify individual cellular compartments, including the cytoplasm and nucleus. NADH and FAD fluorescence is isolated in the cytoplasm. The changes in OMI endpoints are quantified in Figure 3. By 24 hours post excision, the hamster cheek optical redox ratio (NADH/FAD) has increased by 18\% \((p<0.05;\) Fig. 3A). Likewise, the mean NADH fluorescence lifetime is first significantly different \((p<0.05)\) from the \textit{in vivo} values at 24 hours of live-culture (Fig. 3B). No significant difference in mean FAD fluorescence lifetime was observed within the first 24 hours.

3.2 OMI of xenograft-derived organoids

Figure 4. Representative NADH and FAD fluorescence lifetime images of MDA-MB-361 xenograft-derived organoids treated with control IgG and AMG888 + fulvestrant. Each image is 175 \(\mu\)m x 175 \(\mu\)m.
Figure 5. Mean NADH and FAD fluorescence lifetime values of MDA-MB-361 organoid cultures over time.

Representative OMI images of the organoids are shown in Figure 4. OMI resolution is adequately high to image individual cells and identify individual cellular compartments, including the cytoplasm and nucleus. NADH and FAD fluorescence is isolated in the cytoplasm. By 72 hours, cells retained viability and NADH and FAD fluorescence. No significant change in mean NADH fluorescence lifetime or mean FAD fluorescence lifetime was observed (p > 0.05, n = 6) between 24 hours and either 48 hours or 72 hours (Fig. 5).

4. DISCUSSION

Here, we investigated two live-tissue culture techniques for performing OMI on excised tissues. The live-tissue culture technique performed on the living hamster cheek epithelium demonstrates the time-frame during which OMI endpoints from bulk, excised tissue can reflect the in vivo metabolic state. The tumor-derived organoid approach demonstrates a more long-term culture technique (we have grown these organoids up to 1 week with no obvious signs of cell death).

In the live-tissue culture technique, we first observed changes in the optical redox ratio and NADH fluorescence lifetime by 24 hours post tissue removal. No significant changes in the FAD mean lifetime were detected over 24 hours, suggesting that the FAD mean lifetime may be more resistant to metabolic stress caused from excision. IHC analysis of proliferating and apoptotic cells confirmed that the cells were viable up to 48 hours post-excision in this bulk tissue-culture technique. These results suggest the OMI or organoid-processing should be performed on tissue samples within the first 12 hours of excision.

We used a primary tumor-organoid culture technique to sustain longer-lived, more physiologically relevant cultures for testing the therapeutic response of OMI endpoints. The mechanical dissociation, matrigel embedding process enables the tumor macro-suspensions to retain all innate cells and recapitulate lactogenic branching in vitro. Our OMI results show that the organoid cultures retain cellular viability and morphology up to 72 hours post-processing. Further, no significant differences in fluorescence lifetime values were observed over time in these organoid cultures. Development of a live-tissue culture – OMI system would enable OMI of excised tissues, broadening the applicability of OMI. Furthermore, the resolution of OMI is sufficiently high to analyze data on a single-cell level, enabling identification of cells with different metabolic states.

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REFERENCES


