Nonlinear Optical Microscopy and Computational Analysis
of Intrinsic Signatures in Breast Cancer

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Abstract—Recently, new non-invasive imaging methods have been developed and applied to cellular and animal mammary models that have enabled breast cancer researchers to track key players and events in mammary metastasis. Noninvasive nonlinear optical methods such as multiphoton laser scanning microscopy (MPLSM), Fluorescence Lifetime Microscopy (FLIM) and second harmonic generation (SHG) imaging provide an unrivaled ability for obtaining high-resolution images from deep within tissue that can be exploited in the quest to understand breast cancer progression. These optical methods can add greatly to our knowledge of cancer progression by allowing key processes to be non-invasively imaged such as metabolism (on the basis of free and bound NADH detection via FLIM) and interactions with the extracellular matrix (SHG imaging of collagen). In this short application note we present a survey of our latest optical and computational efforts to study intrinsic fluorescence in breast cancer models. In particular we present the latest development in our SLIM Plotter application, an open source visualization program for interactive visualization and inspection of combined spectral lifetime (SLIM) data.

INTRODUCTION

It now is well appreciated that interactions between tumor and stromal compartments play an important role in tumor progression. In order to investigate and understand tumor progression, it is vital to be able to perform complex experiments *in vivo*, including imaging the interaction of tumor cells with host tissues. Numerous changes occur in the stromal cells that are associated with tumors as well as within the stroma itself. For example, tumor-associated fibroblasts have been altered compared to normal fibroblasts, such that these fibroblasts can promote tumor

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formation [1, 2], and cause transformation of even untransformed breast epithelial cells [3]. In addition to changes in stromal cells, the stromal extracellular matrix (ECM) is altered in tumor progression as well. Increased deposition of collagen and other extracellular matrix proteins surrounding tumors, termed desmoplasia, is associated with poor prognosis of several tumor types, including breast, prostate, ovarian, and colon [4-7]. We have demonstrated that changes in collagen density and local concentration can promote adhesive signaling, and enhance cell proliferation and cell migration *in vitro* [8], suggesting that deposition of collagen in the stromal matrix surrounding tumors could have a "feed forward" effect in promoting the progression of tumors. An increase in local collagen deposition is particularly relevant to investigations of invasion and metastasis, as tumor cells migrate through and along collagen matrices [9, 10]. In particular, carcinoma migration *in vivo* occurs along tracks of collagen fibers [11]. *Thus, approaches that allow the visualization of tumor cells in the context of stromal components would be valuable in understanding tumor progression and metastasis.*

Limits of current imaging approaches

Determining the structure of connective tissue (and changes therein) by *in vivo* imaging could impact diagnosis and monitoring of carcinoma progression. However, currently used technologies are hampered by insufficient resolution, lack of specificity, invasiveness, or inadequate statistical power. The need for high-resolution optical imaging is pressing, because current modalities of *in vivo* imaging are restricted in their utility. For example, while significant technological advances have greatly improved the capabilities of ultrasound, MRI, CT, and PET imaging, these modalities are limited to resolutions of ~ 1 mm. However, much higher spatial resolution (1 micrometer or less) is required to visualize structural changes associated with diseased cells and tissues. An understanding of how cancer cells interact with normal tissue environments requires the ability to observe the relationship between subcellular structures, such as focal adhesions, signaling molecules, and components of the ECM within a 3D environment. Some progress has been made imaging EGF-labeled tumor cells with intravital video-microscopy and multiphoton microscopy in order to observe their migratory behavior during invasion within tissues [12-14], however more advances are needed.

Historically, analysis at this anatomical level has been achieved by histology, which involves the removal of tissue, fixing, thin-sectioning, and staining with contrast increasing dyes. While histology remains a "Gold Standard" for pathologists, its interpretation remains highly subjective in that the accuracy depends on the experience and skill of the

interpreting clinician. Furthermore, histological images used to make diagnoses are limited by the spectrum of binding or reactivity of dyes, and do not indicate the endogenous molecules themselves or their assembly.

Moreover, the process of fixing and sectioning tissue potentially destroys certain structures or molecules, and does not allow the investigation of cellular events in real time. For example, the visualization of exogenous carcinoma cells "walking" along collagen fibers has demonstrated the exciting insights that can be gained by observing how cells interact with extracellular matrices [11]. Thus, there remains a clear need for quantitative optical microscopy approaches that can be performed either *in vivo* or on native tissues, thereby eliminating the pitfalls of both non-invasive imaging regimes and traditional histology.

1) Nonlinear optical microscopy

In order to visualize cell-ECM interactions *in vivo*, the optical imaging modalities must have sub-cellular resolution, be able to probe both cellular and tissue structure, and be able to image through several hundred microns of tissues. These goals can also be achieved through the use of nonlinear optical methods coupled with high-resolution microscope imaging schemes. In recent years several biological nonlinear optical microscopy techniques have been developed, including multiphoton excited fluorescence, second harmonic generation (SHG), third harmonic generation (THG), and coherent anti-Stokes Raman scattering (CARS). While two-photon excited fluorescence microscopy have received the most attention, all these methods have all been implemented on laser scanning microscopes for cellular and tissue imaging applications.

These modalities all derive their contrast from different physical principles, however, they share the same crucial attribute that the non-linearity confines the excitation to the desired plane of focus, resulting in reduced phototoxicity over confocal microscopy [15]. The technique is based on the non-linear interactions between light and excitable molecules [16]. In the case of two-photon imaging, the excitation wavelength is set to about twice that of the absorption peak of the fluorophore being observed. If a high-power, ultra-short pulse laser is used, it is possible to achieve instantaneous photon densities that are sufficient to give rise to a significant yield of two-photon events in the focal volume of an objective lens, while maintaining a mean power level that will not damage the specimen. In this manner, fluorophore excitation is confined to the focal volume because the photon density is insufficient to generate appreciable multi-photon events outside of this region. Multiphoton laser-scanning microscopy (MPLSM) exhibits remarkable abilities for obtaining images deep within specimens of poor optical quality because of the relative insensitivity of this technique to the effects of light scattering compared with other optical sectioning techniques such as confocal microscopy.

In addition to intensity measurements, multiphoton excitation (MPE) also works well with fluorescence lifetime imaging [17-19]and spectral imaging [20, 21]. Indeed this is the preferred implementation as MPE eliminates the need for a confocal detection geometry, and results in greater sensitivity (~3-5 fold). This capability is crucial for imaging endogenous fluorophores that have much lower absorption coefficients and quantum yields than engineered fluorescent dyes. Given the similarity in hardware requirements, MPE fluorescence can readily be combined with SHG. SHG is exquisitely sensitive to the structure and organization of fibrillar collagen in tissue [22]. In our studies to study breast cancer invasion and progression, we take advantage of combinations of nonlinear optical microscopy approaches including SHG and MPLSM to perform imaging of in vitro 3D matrices and ex vivo tissues and obtain data not possible with confocal microscopy or other imaging modalities[23].

Second Harmonic Imaging

The extracellular matrix protein collagen has autofluorescent signal components at 325nm and 400nm, which have been attributed to the presence of intermolecular crosslinks between collagen fibrils. A decrease in this fluorescence has been reported just preceding tumor invasion and was attributed to the breakdown of collagen crosslinks by matrix metalloproteinases. Interestingly, collagen fibers also exhibit a strong second harmonic generation signal, which can be exploited to study changes in collagen architecture with respect to cancer progression. SHG results from scattering due to the orientation, polarization and local symmetry found in chiral molecules such as collagen [29]. Both multiphoton induced intensity image data and SHG data can be simultaneously detected on the same multiphoton system. SHG has a forward and backwards component, which can be detected with a transmitted PMT and direct PMT respectively. To date all the observed SHG in our samples has been due to the backwards component, which can be collected simultaneously on our system and separated with a narrow bandfilter (chosen to be ~445nm, which is half the excitation wavelength of 890nm, as is characteristic for the second harmonic).

Using the SHG approach, we were able to define changes in collagen structure associated with tumors visualized in situ[24]. In particular, we noted increased collagen deposition near early pre-palpable tumors, that helps to identify regions undergoing tumor formation; and realignment of the collagen fibers to an angle normal to the tumor boundary, associated with tumor cell invasion. We thus characterized three "Tumor-Associated Collagen Signatures (TACS)," which are reproducible during defined stages of tumor progression, and thus provide standard hallmarks to locate and characterize tumors [13]. These results demonstrate the usefulness of developing SHG approaches in the context of understanding cancer biology.

2) Fluorescent Lifetime Imaging Microscopy (FLIM)/Spectral lifetime imaging microscopy (SLIM)

Fluorescence lifetime is a measure of how long an excited fluorophore stays in the excited state before decay. For strong fluorophores and fully allowed transitions, these

lifetimes are ~1-4 nanoseconds. The actual lifetimes can be affected by local environmental factors such as pH, fluorophore concentration, and binding events. For example, locally high concentrations of a fluorophore result in fluorescence quenching and a decrease in the measured lifetimes. Thus, FLIM has the potential to provide another mode of contrast when imaging unstained samples, and can provide information about the molecular microenvironment. In addition, these measurements can be used to distinguish photons from different fluorophores that have similar wavelengths. Moreover, unlike intensity-based measurements, fluorescence lifetime measurements are absolute and not dependent on intensity.

In addition to using the optical filter approach, which is very effective for separating out fluorophores of known spectra, combined spectral and lifetime imaging (SLIM) can resolve overlapping spectra via analysis of the characteristic lifetimes. This allows the separation of signals with similar excitation wavelengths, but differing in emission spectra, such as the separation of GFP from collagen, or collagen from FAD (see below), all of which are excited in two-photon microscopy at 890 nm.

FLIM and SLIM imaging approaches have great potential for understanding cancer progression. One application is the imaging of endogenous fluorophores. In particular, the metabolic intermediates, FAD and NADH are autofluorescent, and have characteristic spectral properties and lifetime values. Cellular metabolism changes can be determined from the "redox ratio", which is the fluorescence intensity of FAD over the fluorescence intensity of NADH [25]. The redox ratio typically decreases in cancer, and is sensitive to metabolic changes [26]. MPLSM and FLIM can be used to image the redox ratio, and therefore is a useful tool to determine the metabolic activity of cells *in situ.* Moreover, this approach can help differentiate normal from malignant cells. In Bird et al [27] we presented a novel method for deriving functional maps of oxidative cellular metabolism *in vivo* via measurement of the fluorescence lifetimes and the ratio of free to protein-bound NADH using two-photon FLIM [27]. In Conklin et el [28] we use this method to investigate histopathology slides of mouse mammary tissue and found that properties of the fluorescence from the endogenous fluorophores NADH and FAD were indicative of the pathological state of the tissue.

3) SLIM Plotter: Spectral Lifetime Visualization

A major part of our efforts to visualize intrinsic fluorescence with spectral lifetime microscopy is to develop computational tools for visualizing these interactions. SLIM Plotter (Figure 1) is an open-source tool for interactive visualization and inspection of combined spectral lifetime (SLIM) data[31]. The program's main purpose is to be a flexible toolkit for exploration of regions of data collected with a combined spectral lifetime detector, "mining" those regions for meaningful trends.

The program's user interface is divided into two views: a two-dimensional intensity image of each spectral channel,

and a three-dimensional surface plot of lifetime histograms (i.e., exponential decays). SLIM Plotter computes the intensity view by collapsing each lifetime histogram to a single value (i.e., summing the bins), and plots the surface with the X and Z axes corresponding to the histogram's excitation time and photon count, respectively, and the Y axis varying across wavelength. The program also fits 1- or 2-exponential curves to the lifetime data, to determine an approximation of the aggregate lifetime value per channel, using a configurable curve fitting technique—as of this writing, choices include the Levenberg-Marquardt least squares algorithm (LM), and a genetic algorithm.

The limitation of the surface view is that it shows only one decay curve per channel, with no facility for differentiating between spatial locations (pixels) within the image. To mitigate this issue, SLIM Plotter provides a means to select which portions of the data to plot in the 3D window. The program offers two modes of operation: analysis by region, and analysis per pixel. The region-driven mode allows the user to encircle a structure in the intensity window using a freehand drawing tool; the program then sums together the enclosed pixels' lifetime histograms and plots the aggregate surface. The per-pixel mode is more computationally expensive, invoking the curve fitting logic at each individual pixel to compute the lifetime value for each, but allows the intensity image to be recolored according to the computed lifetime values along an adjustable color table. The results for an individual pixel can also be probed and displayed in the surface plot window.

When in per-pixel analysis mode, the program continually works to improve the fit results, attempting to minimize chi-squared error between the fitted curve and the actual data. To minimize needless overprocessing of noise areas, the user can select a region to limit iteration to that region only. In this fashion, the investigator can study lifetime and spectral properties within a specific structure of the sample.

SLIM Plotter is designed to be flexible in its interface and deployment for additional of new algorithms and interfacing with toolkits such as Matlab.

Current SLIM Plotter feature list:

- Read 60+ file formats using Bio-Formats library, including Becker & Hickl SDT files
- Visualize multispectral lifetime histograms as surfaces or lines in 3D for all pixels, individual pixels, or user-selected region of interest
- Report quantities in proper units (wavelength values in nm, time in ns)
- Toggle between perspective or parallel 3D projection modes
- Toggle between linear and log scales
- Align lifetime curve peaks to adjust for hardware skew across spectral channels
- Compute and visualize full-width half max lines for system response
- Perform 1- or 2-component lifetime curve fitting using either genetic or Levenberg-Marquadt (LM)

non-linear least squares algorithms
- Visualize fitted curves or curve residuals simultaneously with acquired data
- Colorize multispectral lifetime surface with a user-defined color table, based on histogram counts, or lifetime values derived from fitted curves
- Fine-grained control over color and Z ranges
- Export results as tab-delimited text file for use in spreadsheet applications
- Detailed log indicating results of operations performed
- Perform per-pixel lifetime computation, with continuous iterative improvement, visualizing the resultant lifetime values spatially

Slim Plotter is available for download at **http://www.loci.wisc.edu/software.**

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