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Motion Compensation for *in vivo* Sub-Cellular Optical Microscopy

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Abstract

In this review we focus on the impact of tissue motion on attempting to conduct sub-cellular resolution optical microscopy, *in vivo*. Our position is that tissue motion is one of the major barriers to conducting these studies along with light induced damage, optical probe loading as well as absorbance and scattering effects on the excitation point spread function and collection of emitted light. Recent developments in the speed of image acquisition have reached the limit, in most cases, where the signal from a sub-cellular voxel limits the speed and not the scanning rate of the microscope. Different schemes for compensating for tissue displacements due to rigid body and deformation are presented from tissue restriction, gating, adaptive gating and active tissue tracking. We argue that methods that minimally impact the natural physiological motion of the tissue are desirable since the major reason to perform *in vivo* studies is to evaluate normal physiological functions. Towards this goal, the methods for active tracking using the optical imaging data itself to monitor tissue displacement and actively move the FOV of the microscope to match the tissue deformation in near real time. Critical for this development was the implementation of near real time image processing in conjunction with the control of the microscope imaging parameters. Clearly the continuing development of methods of motion compensation as well as significant technological solutions to the other barriers to tissue sub-cellular optical imaging *in vivo*, including optical aberrations and overall signal to noise, will make major contributions to the understanding of cell biology within the body.

Introduction

In recent years there has been a growing interest in the application of optical microscopy techniques to evaluating sub-cellular events, *in vivo*. This interest has been driven by many factors. One of the overriding issues is the realization that many cellular events observed in culture, the classical environment for intra-vital cell biology studies, are severely limited by the nature of the culture process itself which modifies the behavior and programming of the cells and lacks the physiological interactions of the complex tissue structures *in vivo*. In addition, the technology to perform high resolution optical imaging studies, *in vivo*, has been progressively improving. Herein, we will discuss the motives for performing intra-vital microscopy studies and relate these driving forces to one of the major barriers to these studies, tissue motion. We apologize in advance for any omissions in our citations in this brief review as this field has been developing so quickly that we are certain that some oversights occurred. It is also important to note that the motion compensation field in imaging is enormous from astronomy, remote monitoring, medical imaging, manufacturing

and process monitoring to simple photography thus the reader is encouraged to explore these other fields in searching for solutions to their specific problems. Indeed, most of the solutions discussed below have been derived from approaches used in other fields.

Why perform an *in vivo* optical microscopy study? Surprisingly, this question is often not dealt with at the beginning of a research study even in more macroscopic studies such as MRI or CT (Balaban & Hampshire, 2001). That is, if you are just interested in high resolution anatomy, receptor site distributions, vascular structure, etc. these studies should be conducted e*x vivo* under optimal imaging conditions with no motion, time or tissue access restraints. The recent explosion of super resolution optical studies on fixed samples underscores this point. Without question, the resolution and information content of *ex vivo* studies far exceed the more complicated *in vivo* studies. Thus, the reasons for conducting an *in vivo* study should be compelling to compromise the microscopy data content. In general, the processes that require *in vivo* microscopy monitoring are observations of the physiological behavior of tissues and cells in its true complex environment. These types of studies include the evaluation of events such as the regulation of blood flow, cell-cell or intracellular signaling events, such as orchestration of brain activity, systemic hormonal regulation, cellular motility, developmental biology, etc. Finally, the optical imaging experiment can also be used to specifically alter the cell and perturb the system in a controlled manner via caged compounds or genetically programed optically triggered ion channels (Llewellyn *et al*., 2010). Thus, the behavior of the imaging experiment *over time* is usually a critical element for *in vivo* microscopy.

Motion Composition

There are numerous barriers to the application of high resolution optical imaging to mammalian systems, *in vivo*. Some of these might include the depth of penetration (tissue access, scattering, absorbance, optical aberration etc.), sensitivity, probe specificity and compartmentalization as well as light induced damage. We take the position that tissue motion is currently the major barrier to successful sub-cellular microscopy, *in vivo (*Schroeder et al., 2010, Bakalar et al., 2012). On the micron scale tissue motion is continually occurring due to alterations in blood pressure, ventilation, water redistribution (usually associated with inflammation associated with surgery to gain access to the region of interest) and numerous the physiological process such as muscle contraction or water redistribution due to epithelial function. Regrettably, photo-damage is also a too common source of motion; however we often use this type of motion to provide an early marker of this destructive process. The complications of motion for microscopy are perverse and impact numerous features from resolution, signal to noise (through reduced signal averaging), to limiting longitudinal studies on given regions of the tissue. Motion can be divided into two time domains, rapid perturbations that occur on the order of image framing rates and slower motions, or drift, that are common for most applications and with many physiological perturbations. The physical outcome of sample motion during image acquisition may either be a rigid body transformation (translation and rotation only) or a non-rigid body deformation (includes shear, extension and extension in addition to rigid body transformation) (Schroeder et al., 2010) requiring different compensation mechanisms. The non-rigid body deformation displacements are very significant on the micron scale in

biological tissues as they are generally very compliant; however, the scale of the deformation is relatively small relative to the rigid body displacements were most of the compensation efforts have been applied.

Motions occurring on the order of the imaging frame rate are the most difficult to correct for since the structure is changing before the full image can be collected. This class of motion includes muscle and non-muscle actin-myosin contraction activity, structures in and around arterial blood vessels or in the region of the lung respiratory activity. This class of "motions" might include rapid cellular signaling events such as calcium or electrical transients. If the motion is physiological and cannot be eliminated, as will be discussed below, then the options are limited. The easiest method to speed up acquisition is to reduce the field of view (FOV) (i.e. single line scan as an extreme). If the motion is extremely reproducible, which is very rare on the micron scale in contrast to millimeter MRI or CT space scales, gating (Taylor *et al*., 2011, Vinegoni *et al*., 2014) or even more efficient gating with view sharing (Lee *et al*., 2012, Foo *et al*., 1995, Vinegoni et al., 2014) could be used to stitch together rapidly collected different small FOVs collected in a gated fashion to create a larger FOV image or time course. In addition this approach can be used to increase the image signal-to-noise ratio (SNR) by time averaging of a small volume without tiling. Again gating is only viable for subcellular imaging if the motion is nearly completely reproducible on the micron scale, a regrettably rare event in most *in vivo* preparations.

Recent developments have generated remarkable improvements in full frame imaging speed from resonant scanning systems and wide field schemes almost on a monthly basis (Holekamp *et al*., 2008, Seo*et al*., 2013, Goda *et al*., 2009) coupled to extremely efficient detection systems, such as electron multiplying charge coupled devices, and efficient photon collection schemes (Combs *et al*., 2013). From these developments it is clear that the fundamental number of detectable photons that can be generated from a micron or sub-micron voxel is rapidly becoming the limiting factor in speed of acquisition of high resolution images (Holekamp et al., 2008). Thus, the imaging speed is often limited by the SNR rather than the scanning speed of the microscopes for many applications. To increase speed, the SNR of the probes must be improved by enhancing the fluorescence efficiency or effective concentration probes. In the area of fluorescence efficiency, the field is approaching efficiencies of close to 1 for several probes, such as nano-diamonds or quantum dots, leaving little room for improvement. With current high powered lasers and high NA lenses increasing the excitation light power is limited by light induced damage and photo-bleaching (for example see (Carlton *et al*., 2010)). One method to improve this situation is to move more into the infrared excitation region avoiding the higher energy photons more damaging to tissues using multi-photon excitation schemes, possibly even using three photon excitation (Yu *et al*., 2013, Wokosin *et al*., 1996), as well as infrared molecular probes. Solutions might include increasing the number of emitters on each nano-probe; however, this is simply a linear improvement and will quickly generate steric problems. Another approach would be to use modulation techniques to shift the measurement away from frequencies with high noise sources or better, to temporally modulate the emission of the probe independently from the background signals to eliminate DC background signals, improving the system SNR. On example of this exciting approach is the used of oscillating

magnetic fields on nano-diamonds (Igarashi *et al*., 2012). In summary, the major technological improvements in increasing imaging speed to freeze biological motion or physiological events has driven the field forward however, without significant improvements in several areas including limiting light induced tissue damage, for example see (Editorial, 2013), increasing signal detection or probe emission efficiencies, SNR will now fundamentally limit the speed and associated resolution of *in vivo* microscopy moving forward.

Motion occurring slower that the framing rate is also problematical since it precludes following longitudinal events in the tissue, again a major reason for doing *in vivo* microscopy. In addition, this type of motion can prevent the image averaging generally required to improve the SNR of the intra-vital experiment using minimal peak power. This is especially true when spectroscopy is required to differentiate the position or chemical state of different optical probes that significantly reduces photon collection efficiency.

Solutions for compensating for tissue motion in general vary from those that attempt to restrict motion, via cover glass placements, suction devices or various types of tissue clamp that restrict the motion of the tissue. Two of the most successful preparations in this area is imaging in the brain using stereotaxic devices with glass or thinned bone viewing windows, for example see (Yang *et al*., 2010), have proven to be a useful preparation, but again motions on the order of microns are still routinely observed over time. Another approach is the rigidly clamped hamster cheek pouch muscle extensively studied for vascular dynamics (Duling, 1973). Even with these preparations local motion and deformation often restricts the resolution of these experiments to the cellular and not sub-cellular level. Due to the potential physiological perturbation induced by the restriction of motion affecting everything from regional blood flow to distorted anatomy, we prefer to use retrospective motion or active prospective tracking compensation techniques minimizing the impact of the experiment on the physiology of the tissue.

The simplest motion compensation scheme consists in collecting a large 3D volume that is larger than the motion displacement occurring over the experiment time course and retrospectively correcting for rigid body and deformation in a retrospective manner. Usually this is done by selecting a reference volume and performing 3D cross correlation analysis to re-register the image volumes over time for longitudinal studies or permit image averaging. This is very effective in providing a correction for the drift and/or deformation of the tissue over time. Another approach that works well with simple focus plane motion is tracking or adaptive tracking by moving the focus alone either physically or with deformable mirrors (Poland *et al*., 2008). These 3D or 2D cross-correlation approaches require high SNR and excellent spatial frequency information to successfully register the volumes. In general the registration will only be as good as the SNR and spatial frequency information available in the images. We have found that labeled vascular images collected in parallel with cellular studies provides excellent SNR images with good spatial characteristics (i.e. high spatial frequency content) to track on (Bakalar et al., 2012, Schroeder et al., 2010). Recently, we have also found that nuclear stains, such as SYTO dyes that can be loaded *in vivo* also provide good FOV coverage, SNR and spatial frequency information for motion compensation.

Though the rapid collection of a 3D volume and retrospective registration is a very simple and straightforward process; however in our experience motion is usually much greater than a reasonably sized 3D volume that can be collected with micron resolution. Rigid body multi-dimensional displacements sometimes exceeding a millimeter are observed *in vivo* with metabolic perturbations, post exercise, vascular volume changes or hormonal stimulations of different types (Bakalar et al., 2012, Schroeder et al., 2010). Thus, in most cases we find that active motion tracking by physically moving the stage and objective so as to keep a given FOV within the volume collected is a more effective approach. We accomplish this task by using a fast resonant imaging system for image collection coupled to a real time GPU based computer system that calculates the 3D cross correlation to determine the rigid body displacement in near real time. This information is then rapidly used to move the tissue using a fast high precision microscope stage securely coupled to the preparation. Using this approach with rather standard computational capabilities, the FOV can be maintained on the micron scale with rigid body displacement velocities approaching 200 microns/min (Bakalar et al., 2012). This velocity is well within the range of the displacement rates we observe in many *in vivo* tissues (Rothstein *et al*., 2005, Rothstein *et al*., 2006, Schroeder et al., 2010, Bakalar et al., 2012). Since active tracking is only a rigid body correction a retrospective 3D deformation correction is also required to register the images for analysis and signal averaging (Schroeder et al., 2010). Again, the deformations are usually small and do not result in significant displacement out of the FOV requiring active tracking. It is also important to recognize that the displacement data obtained from tracking actually provides useful information on the physiological and cellular response to perturbations, thereby expanding the information collected in these studies. We believe that the type of real time image processing and interpretation that permitted the active tracking discussed above or guide rapid tiling of multi-FOV volumes across a tissue (Schroeder *et al*., 2011) will greatly improve the efficiency of intra vital imaging moving forward.

In summary, we have focused on the influence of motion on *in vivo* subcellular imaging. Motion is a constant obstacle in acquiring high resolution longitudinal data *in vivo* due to the high compliance and dynamic nature of biological tissues. Imaging speed to freeze rapid motions has quickly approached the SNR limitations of many optical imaging approaches suggesting that improvements in SNR performance are required to gain optimal benefits from the rapid scanning techniques. Many new promising approaches are being developed to improve both the speed and SNR of detection. Techniques have been developed to compensate for rigid body transformations by using proper spatial reference labeling and 3D cross-correlation techniques and are robust enough to cope with modest non rigid body deformation displacements. Using near real time computational approaches in parallel with rapid image acquisition schemes, prospective motion compensation schemes have resulted in systems that can compensate for deformation velocities as high as 200 microns/min. Further development in imaging speed, probe SNR detection and real time computational systems could easily improve motion compensation schemes improving the utility of *in vivo* microscopy.

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