NOTE

Effects of formalin fixation on tissue optical polarization properties

M F G Wood[1]**, N Vurgun**[1]**, M A Wallenburg**[1] **and I A Vitkin**[1,2]

[1] Division of Biophysics and Bioimaging, Ontario Cancer Institute and Departments of Medical Biophysics
[2] Radiation Oncology, University of Toronto, 610 University Avenue, Toronto, ON M5G 2M9, Canada

E-mail: mwood@uhnres.utoronto.ca

Abstract

Formalin fixation is a preparation method widely used in handling tissue specimens, such as biopsies, specifically in optical studies such as microscopy. In this note, we examine how formalin fixation affects the polarization properties of porcine myocardium and liver as assessed by optical polarimetry. Spatial maps of linear retardance and depolarization were derived from four myocardial and four liver samples before and after formalin fixation. Overall, linear retardance and depolarization increased after fixation for both myocardium (15% and 23% increase, respectively) and liver (38% and 51%, respectively). The relative increase in retardance was greater in liver compared to myocardium, although the absolute increase in retardance was comparable for both. The effect of fixation on bulk optical properties was also investigated for myocardium where the scattering coefficient increased from 92 to 132 cm^{-1} and the absorption coefficient remained constant at 1.1 cm^{-1}.

(Some figures in this article are in colour only in the electronic version)

1. Introduction

Polarized light can be used as a diagnostic tool to characterize tissue structure (Wu and Walsh 2006, Ghosh *et al* 2008a, 2008b, Wood *et al* 2009), provided sufficient polarization is maintained in polarized light interacting with the biological sample, so that one can quantify the tissue polarization effects of the medium. Linear birefringence and optical rotation are two useful polarization properties that provide potentially useful biological metrics. As an example, we have demonstrated linear birefringence as a metric for measuring the structural organization of the myocardium in healthy, infarcted, and stem-cell-regenerated tissues (Wood *et al* 2009). In addition, we have investigated the use of glucose-induced optical rotation to quantify glucose concentrations in tissue-like media (Guo *et al* 2006).
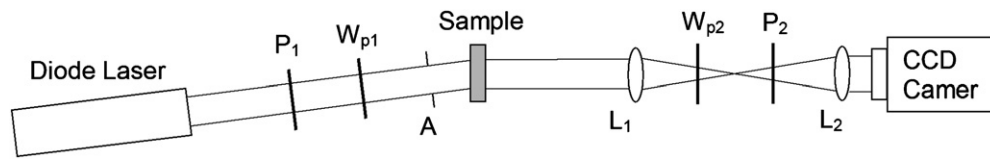
Figure 1. Schematic of the experimental optical polarimetry imaging system: P_1 and P_2, linear polarizers; QWP_1 and QWP_2, removable quarter-wave plates; and L_1 and L_2, lenses.

When preparing samples for polarized-light-based *ex vivo* tissue studies, it is often advantageous to fix samples in formalin. In addition to arresting the decay process, fixation offers easier handling, prolonged storage, precise sectioning of tissues to uniform thickness, and the possibility of combining optical polarimetry with histology. A popular choice of fixative is formalin (10% formaldehyde). Formalin is a crosslinking agent that acts at the molecular level by linking together soluble and structural proteins (Abe *et al* 2003). While fresh tissues appear visibly different under white light when compared to fixed tissues, there are very few studies that compare the optical properties (scattering and absorption coefficients) of fresh and fixed tissue (Hsiung *et al* 2005) and no studies that compare the optical polarization properties. Due to the advantages that formalin fixation provides in optical polarimetry studies, and the fact that it has been employed in previous studies (Wood *et al* 2009), it would be prudent to measure and quantify how formalin fixation influences tissue birefringence and depolarization properties. The magnitude of these changes can then be used to test the validity of polarimetric results obtained in previous studies from formalin fixed tissues. This is particularly important in the context of the technique's potential *in vivo* translation, as it must be verified if the trends measured in fixed tissue are also present in fresh and living tissues as well.

The outline of this note is as follows. We begin by introducing the polarimetry imaging system employed in this study and the polar decomposition method used for analyzing the data. We then discuss formalin fixation and sectioning methods for both porcine myocardial and liver tissue. Next, we present measured birefringence and depolarization for samples before and after fixation and discuss the implications. These changes in polarization properties are related to changes in bulk optical properties (scattering and absorption coefficients). We conclude with a summary of our findings and evaluate the suitability of formalin fixation in tissue optical polarimetry studies.

2. Methods

2.1. Polarimetry imaging system and analysis

An imaging polarimetry system was employed in this study to allow for spatial mapping of polarization measurements (Wood *et al* 2010). The experimental set-up is shown in figure 1. The input beam at 635 nm from a diode laser (Thorlabs, Newton, NJ) is passed through a linear polarizer and removable quarter-wave plate to control the input polarization state (quarter-wave plate in place when circular polarized light is required). Next, the beam passes through a lens and an iris of approximately 1 cm diameter before interacting with the sample, a portion of the scattered light being detected by the analyzing optics. These consist of a lens to focus the transmitted light through another removable quarter-wave plate, a second linear polarizer, and a third lens (quarter-wave plate in place when circular polarized light detection is

required). The light is captured by a CCD camera (CoolSNAP K4, Photometrics, Tucson, AZ) and stored as a 2048×2048 pixel intensity image ($7.4 \times 7.4 \, \mu m^2$ pixel size and $2 \times 2 \, cm^2$ field of view).

The sample is mounted between the input and the analyzing optic in an off-axis forward direction at $35°$ angle. This allows sufficient polarization to be detected; while at the same time prevents collimated transmission light from saturating the CCD.

The polarization of the input and output light was tracked using the Stokes vectors ($S = [I \ Q \ U \ V]^T$), where I is the intensity of an input beam, Q and U correspond to the intensities of linear polarizations, and V corresponds to the intensity of circular polarization. For each of four input polarization states (horizontal linearly polarized (H), vertical linearly polarized (V), $+45°$ linearly polarized (P), right-handed circularly polarized (R)), the output Stokes vectors were measured.

Mueller matrices (polarization transfer function of the medium) were then calculated for each sample using the output Stokes vectors for the input states H, P, V and R (Ghosh et al 2008b). In order to extract the polarization parameters (in this case birefringence and depolarization), polar decomposition was performed on the Mueller matrices (Lu and Chipman 1996). This decomposition method is required as the effects of the medium influence the polarization of the light simultaneously, thus complicating the quantification of the parameters individually.

Birefringence is a property of a material exhibiting different refractive indices along different directions; the difference in these indices Δn is termed the birefringence. Highly organized (anisotropic) biological media such as collagen (Canham et al 1990) and myocardium (Schoenenberger et al 1998) exhibit higher birefringence compared to more homogenous (isotropic) media such as liver and brain. These birefringent tissues are generally assumed to be uniaxial (refractive index differs only along one direction) and based on measurements to have higher refractive indices along the direction of birefringence (orientation of the collagen or muscle fibers). When propagating through birefringent media, linear polarizations along these directionally varying refractive indices will propagate at different speeds and thus undergo a phase shift. This phase shift can be quantified as the retardance δ of the polarization after propagating through the medium. Retardance can be expressed as $\delta = 2\pi \Delta n d/\lambda$, which relates the change in phase δ to the birefringence Δn, where d is the optical path length, and λ specifies the wavelength of the light. Since retardance δ is directly proportional to birefringence Δn, it can be used as a measure of the anisotropy of the sample if the optical pathlength d is held constant, and can be used as a diagnostic tool to assess tissue microstructure and organization. When propagating through scattering media, such as biological tissue, the polarization of light becomes randomized through multiple scattering (and potentially through birefringence effects). The randomization leads to an overall depolarization of the incoming beam. Using the elements of the Mueller matrices obtained from the decomposition for depolarization, $\mathbf{M}_\Delta$, and retardance, $\mathbf{M}_R$, the tissue retardance and depolarization can be quantified as

$$\delta = \cos^{-1}(\sqrt{(\mathbf{M}_R(2, 2) + \mathbf{M}_R(3, 3))^2 + (\mathbf{M}_R(3, 2) + \mathbf{M}_R(2, 3))^2} - 1), \qquad (1)$$

and

$$\Delta = 1 - \left(\frac{|tr(\mathbf{M}_\Delta)|}{3} \right), \qquad (2)$$

where tr is the trace of the matrix, and the indices following the matrices indicate the element of the 4×4 matrix. The matrix decomposition and values of δ and Δ are calculated for each pixel measured by the imaging system and then averaged over all the pixels from the sample.

2.2. Sectioning and formalin fixation of myocardium and liver samples

In order to study how fixation affects different types of tissue, we chose a porcine animal model and examined two types of tissue: highly birefringent (anisotropic) myocardium and low birefringent (isotropic) liver, to gain insight into the effects of formalin fixation on these differing tissue types. Fresh porcine hearts and livers were obtained from a local butcher and shortly after sectioned into approximately 1 mm thick slices using a scalpel. Heart samples ($n = 4$) were cut axially from the left ventricle and liver samples ($n = 4$) were cut parallel to the liver surface.

After imaging fresh samples in the polarimeter, the samples were fixed immediately in 10% formalin solution. Following fixation, the same samples were imaged again 1 day and 6 days post-fixation.

3. Results and discussion

The retardance and depolarization images for the heart and liver samples were averaged for each image to generate mean retardance δ and depolarization Δ values for each sample. This averaging was necessary to compare the values before and after fixation, as pixel-by-pixel comparison of the values was impractical due to the differing positions and orientations of samples between measurements. The variations in spatial retardance values are due to variations in the orientation of muscle fibers through the myocardium. Before fixation the mean retardance values were $178° \pm 85°$ and $18° \pm 3°$ for the fresh heart ($n = 4$) and liver ($n = 4$), respectively. This demonstrates that myocardial tissue is highly anisotropic and organized, as revealed by its high birefringence. The large variance of values for myocardium is likely due to variations in tissue thickness as sectioning was performed by hand. In contrast, liver tissue is relatively isotropic, exhibiting low birefringence. The mean depolarization values before fixation were $52 \pm 6\%$ and $57 \pm 6\%$ for the fresh heart and liver, respectively. This demonstrates that both heart and liver are significantly depolarizing media.

After 6 days fixation the mean retardance values increased to $194° \pm 75°$ and $24° \pm 2°$, for the heart and liver, respectively. The mean depolarization also increased after 6 days fixation to $64\% \pm 7\%$ and $85\% \pm 5\%$, respectively. Representative retardance and depolarization images for both heart and liver, before and after fixation, are shown in figure 2. A modest overall increase in both retardance and depolarization values is evident after fixation; however, the increase in depolarization is greater than that for retardance.

To examine how δ and Δ change for each individual sample following fixation, the relative percent increase in δ and Δ were calculated relative to the fresh state of each sample. The percent changes were then averaged across the samples for both liver ($n = 4$) and heart ($n = 4$). This use of relative percent change is important, as sectioning variations of fresh tissues make the direct comparison of absolute measurement values of retardance and depolarization between samples problematic. The percent change for retardance and depolarization are shown in figure 3.

Retardance was found to increase following formalin fixation for both myocardium and liver. The average relative increase in δ for the heart and liver following overnight formalin fixation was found to be $14 \pm 10\%$ and $25 \pm 22\%$, respectively. After 6 days fixation, the relative percent increases in δ were calculated to be $15 \pm 16\%$ and $38 \pm 15\%$. While the relative increase is larger for the liver compared to heart, it should be noted that the liver exhibits a low birefringence signal initially compared to the heart ($178°$ for the heart compared to $18°$ for the liver). Due to the low underlying birefringence of the liver, small increases in the magnitude of δ above this low baseline lead to large relative percentage increases in δ.
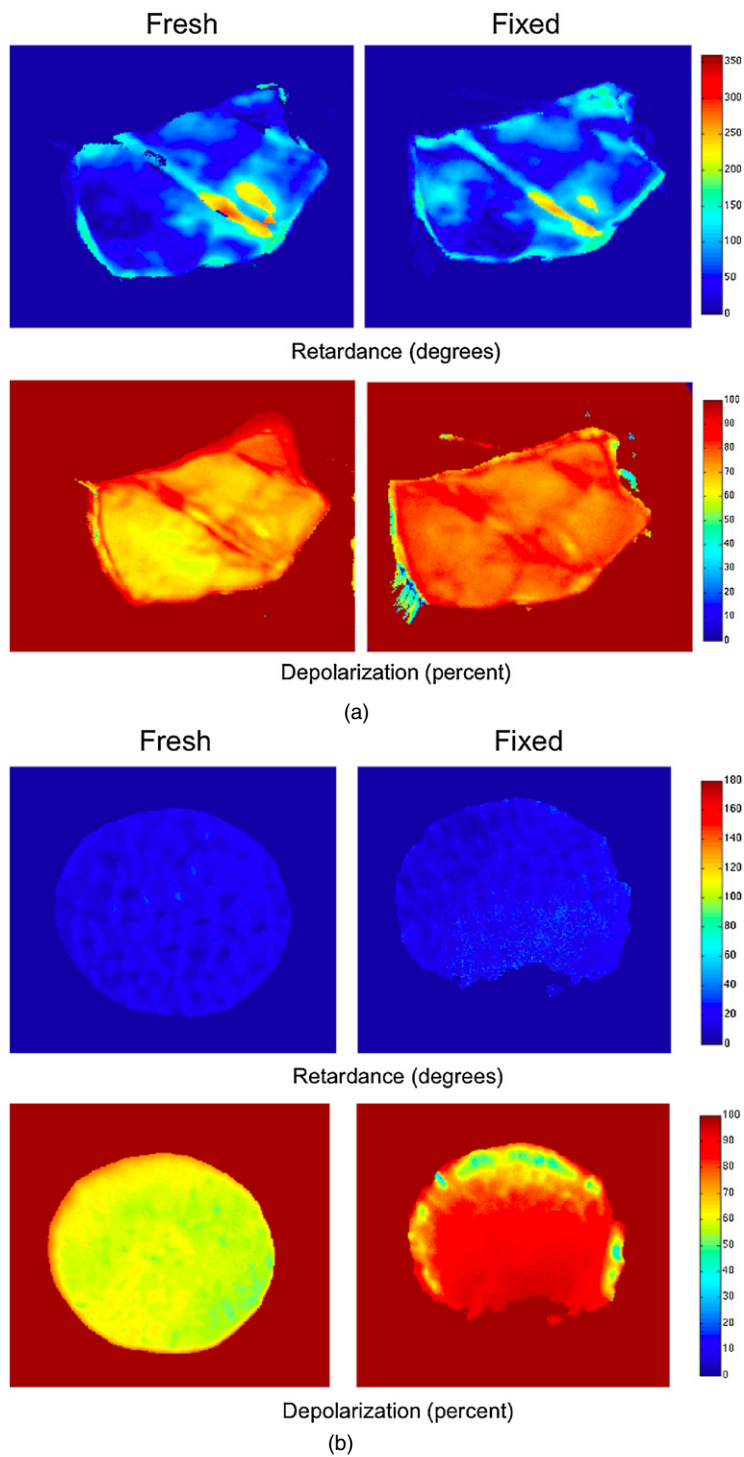
Figure 2. Retardance and depolarization images for representative myocardium (a) and liver (b) samples ($n = 1$ out of 4 samples for both myocardium and liver). Note the increases in both retardance and depolarization after fixation for both types of tissue.
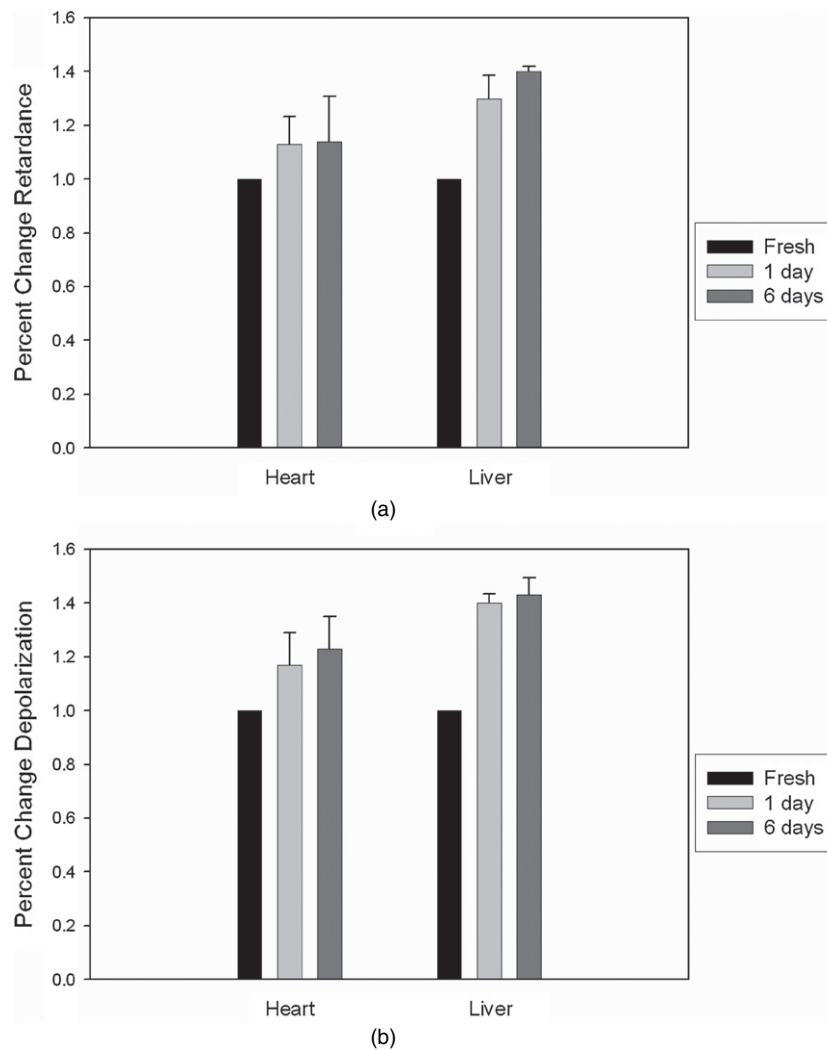
Figure 3. The average of the percentage increases for heart ($n = 4$) and liver ($n = 4$) in (a) retardance and (b) depolarization values. The spread is given by 1 standard deviation.

The average relative increase in Δ for myocardium and liver following overnight formalin fixation was found to be $17 \pm 4\%$ and $49 \pm 12\%$, respectively. After 6 days fixation, the relative percent increases in Δ were calculated to be $23 \pm 3\%$ and $51 \pm 7\%$, respectively.

The increases in both retardance and depolarization post-fixation can in part be attributed to a volume increase (measured by submerging the samples in water and measuring the volume displacement), resulting in a longer optical pathlength. However, only a small volume increase (4%) was measured after fixation. The increases in retardance and depolarization may also be due to changes in bulk optical properties. Previous studies have measured an increase in optical scattering after fixation, due to the cross-linking of proteins creating a more highly scattering media (Hsiung *et al* 2005). This was confirmed using double-integrating sphere measurements (Pickering *et al* 1993) on myocardial tissue. The scattering coefficient (μ_s) at

635 nm was measured to increase from 92 cm^{-1} in fresh tissue to 132 cm^{-1} with fixation, while the absorption coefficient remained constant at 1.1 cm^{-1}.

This increase in scattering accounts for the increase in depolarization, as it causes additional randomization of the input light's polarization. The increase in scattering may also be responsible for the increase in retardance, as resultant longer average pathlengths increase the effect of birefringence yielding greater retardance. However, the increase in retardance may also be due to the cross-linking of proteins, forming a more anisotropic tissue structure displaying increased birefringence. At present, the cause for the increase in birefringence is uncertain, and is likely due to a combination of both mechanisms.

The fact that we see some small changes in polarization parameters is perhaps not surprising. Changes in bulk optical properties have previously been reported (Hsiung *et al* 2005); polarimetry explores other aspects of light–tissue interactions, specifically the changes in light polarization induced by tissue, and hence its derived metrics will also likely be affected. The large relative percentage increases in both depolarization and retardance in liver following fixation versus the smaller percentage changes in myocardium suggest that the response of tissues to formalin fixation may be tissue dependent. Based on these results we suggest that the changes in polarization properties with formalin fixation should be measured for a specific tissue type prior to engaging in polarimetry studies with fixed tissues.

4. Conclusions

The effect of formalin fixation on measured polarization parameters was studied using optical polarimetry in porcine tissues. The outcome of this study shows that both highly birefringent (anisotropic) myocardium and low birefringent (isotropic) liver samples exhibit higher retardance and depolarization following formalin fixation. The magnitude of change in retardance was relatively small for both myocardium and liver ($\sim$10% and 30% increase, respectively). The depolarization of light was also found to be higher for formalin-fixed tissues (25% and 50% increase for myocardium and liver, respectively). From these results, we conclude that for porcine myocardium and liver, the changes in tissue birefringence are relatively small; however, different magnitudes of the changes suggest that formalin fixation effects each tissue organization in a different manner. Similarly, the effect of fixation on depolarization for porcine myocardium and liver are more significant, and differ considerably between the two examined tissue types, again indicating differing effects due to fixation. These results suggest that the use of formalin fixation for *ex vivo* polarimetry studies is acceptable as it does not radically change the polarization properties. However, we would advise investigating the polarizing effects of fixation on the specific tissue type.

References

Abe M, Takahashi M, Horiuchi K and Nagano A 2003 The changes in crosslink contents in tissues after formalin fixation *Anal. Biochem.* **318** 118–23

Canham P B, Talman E A, Finlay H M and Dixon J G 1990 Medical collagen organization in human arteries of the heart and brain by polarized light microscopy *Connect. Tissue. Res.* **26** 121–34

Ghosh N, Wood M F G, Li S, Weisel R D, Wilson B C, Li R and Vitkin I A 2008a Mueller matrix decomposition for polarized light assessment of biological tissues *J. Biophoton.* **2** 145- 146

Ghosh N, Wood M F G and Vitkin I A 2008b Mueller matrix decomposition for extraction of individual polarization parameters from complex turbid media exhibiting multiple scattering, optical activity, and linear birefringence *J. Biomed. Opt.* **13** 044036

Guo X, Wood M F G and Vitkin I A 2006 Angularly-resolved linear stokes polarimeter measurements of light scattered by turbid chiral media *J. Biomed. Opt.* **11** 041105

Hsiung P H, Nambiar P R and Fujimoto J G 2005 Effect of tissue preservation on imaging using ultrahigh resolution optical coherence tomography *J. Biomed. Opt.* **10** 064033

Lu S Y and Chipman R A 1996 Interpretation of Mueller matrices based on polar decomposition *J. Opt. Soc. Am. A* **13** 1106–13

Pickering J W, Prahl S A, Van Wieringen N, Beek G F, Sterenborg H J C M and Van Gemert M J C 1993 Double-integrating-sphere system for measuring the optical properties of tissue *Appl. Opt.* **32** 399–410

Schoenenberger K, Colston B W, Maitland D J, Da Silva L B and Everett M J 1998 Mapping of birefringence and thermal damage in tissue by use of polarization-sensitive optical coherence tomography *Appl. Opt.* **37** 6026–36

Wood M F G, Ghosh N, Li S, Weisel R D, Wilson B C, Li R and Vitkin I A 2009 Polarized light based birefringence measurements for monitoring myocardial regeneration *Proc. SPIE* **7179** 719108

Wood M F G, Ghosh N, Wallenburg M A, Li S, Weisel R D, Wilson B C, Li R and Vitkin I A 2010 Polarization birefringence measurements for characterizing the myocardium, including healthy, infracted, and stem-cell-regenerated tissues *J. Biomed. Opt.* **15** 047009

Wu P J and Walsh J T Jr 2006 Stokes polarimetry imaging of rat tail tissue in a turbid medium: degree of linear polarization image maps using incident linearly polarized light *J. Biomed. Opt.* **11** 014031