Fixative Induced Effects in Labeled and Unlabeled Fluorescence: Implications for Biomedical Imaging Studies

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Background

Fluorescence is the emission of light by certain molecules or structures occurs when they are energetically excited by incident light of specific wavelength (Fig. 1). This phenomenon is especially prominent in various biological samples, such as cells, tissues, and organisms [1], [2]. Fluorescence is observed and studied in a large scope of biological materials and is produced by both natural-occurring (**endogenous**), and externally introduced (**exogenous**), fluorophores [2]. Tissue fluorescence is a valuable tool to shed light on underlying biological mechanisms and is also promising as a potential diagnostic marker. Both endogenous and exogenous fluorophores encode vital information regarding properties and composition of tissue samples [1].

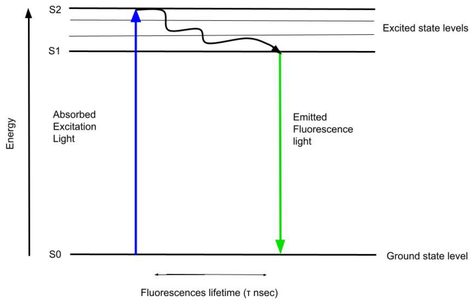

Figure 1. Energy diagram of fluorescence.

In many biological studies, as well as clinical practice, tissues and cells often undergo fixation[3]. Fixatives preserve biological samples while preventing contamination by crosslinking proteins and other biological molecules through chemical methods to maintain their structural integrity, molecular composition, tissue and cell morphology, and ensure a low health risk experiment by eliminating infectious materials [3], [4]. Ultimately, fixing tissues can facilitate long-term use, storage, and repeatable experimentation [4]. Although fixation is required for histology, it has been documented that chemical fixation can cause alterations in the fluorescence properties of exogenous and endogenous fluorophores, which are valuable markers for understanding biological processes, ultimately reducing the accuracy and reliability of quantitative fluorescence measurements [5], [6].

Objective

Our primary objective is to investigate the effects of Paraformaldehyde (PFA) fixation on endogenous fluorophores and exogenous fluorophores' resulting fluorescence intensities compared to phosphate buffered saline (PBS) through multispectral fluorescence imaging (MFSI).

Methods

To evaluate the effects of **PFA** on tissue fluorescence, we imaged brain tissue samples using **MSFI** from two cohorts of mice (n= 6 each): the SOX10 Cre; R26R-Brainbow 2.1/Confetti treated (TRT) mice (expressing four exogenous fluorophores), and wild type Cre-negative controls. Specimens from each were immersed in 10 ml of PFA or **PBS** as a control. The fluorescence intensity was captured using MFSI every 15 minutes over three hours. The resulting images underwent analysis to generate quantitative metrics for the fluorescence signal. This was achieved by computing the average pixel values over specified Regions of Interest (ROIs).

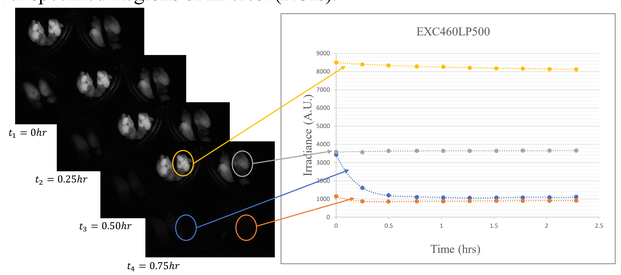

Figure 2. Visualization for imaging and quantification process. Regions of interest were isolated and the fluorescence signal over time was measured for each sample.

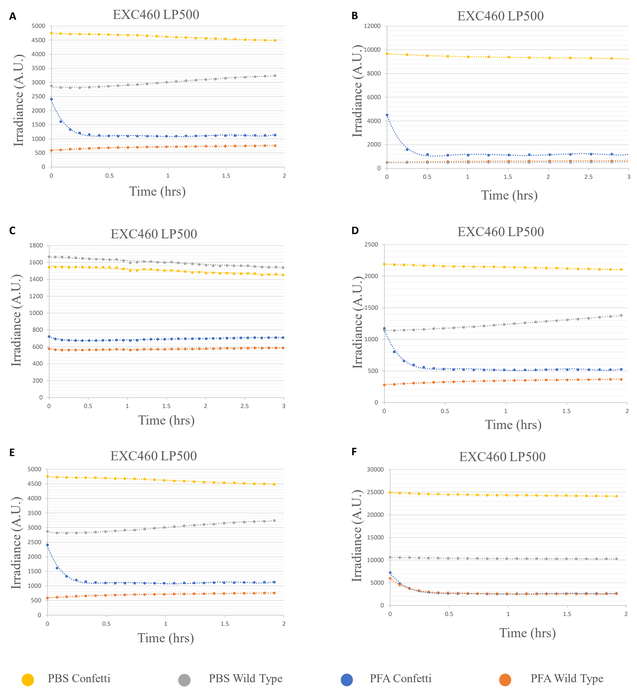

Figure 3. Fluorescence intensity time series | 460nm Excitation, 500nm Long-Pass - for data trials one through six A – F. We see that… EXC: Excitation filter [nm], LP: Long Pass filter [nm].

Results

Figure 4. Fluorescence intensity quantification for different excitation and emission wavelengths. (A-D) Irradiance Ratio 0hr:0.5hr, . EXC: Excitation filter [nm], LP: Long Pass filter [nm]. (E-H) Irradiance Ratio 1hr:3hr, . EXC: Excitation filter [nm], LP: Long Pass filter [nm].

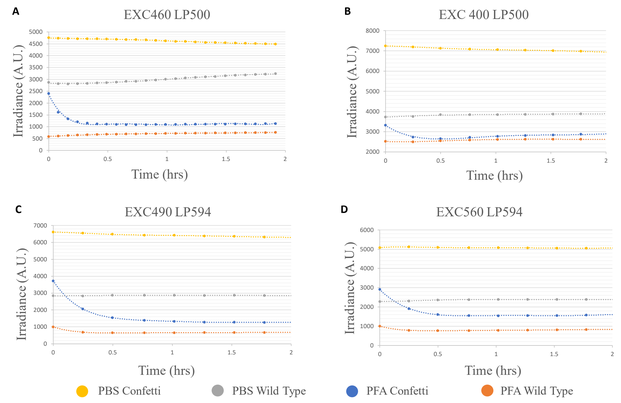

Figure 5. Fluorescence intensity time series for different excitation and emission wavelengths for one data trial. EXC: Excitation [nm], LP: Long Pass [nm].

Conclusions

Looking at these graphs we notice four distinct characteristics for all four 'EXC' and 'LP' combinations:

Wild Type in PFA
- Mean multiplicative decay factor of -0.139 in fluorescence seen within the first half hour.
- Mean multiplicative growth factor of 0.0376 in fluorescence seen from the first hour to the final hour.

Wild Type in PBS
- Mean multiplicative growth factor of 0.0172 in fluorescence seen from the first hour to the final hour.

Confetti Brainbow in PFA
- Mean multiplicative decay factor of -0.4766 in fluorescence seen within the first half hour.
- Mean multiplicative growth factor of 0.0511in fluorescence seen from the first hour to the final hour.

Confetti Brainbow in PBS
- Exhibited fluorescence values greater than WT in PBS.
- Average decay multiplicity factor of -0.0561 in fluorescence seen from the first hour to the final hour.

We can conclude that optimizing operational constraints such as fixation time is necessary in tissue processing. From our results we see that the **PFA fixative does alter the fluorescent properties of the tissue** when performing fluorescence imaging. **The PFA quenches or reduces the fluorescence signa**l, leading to decreased sensitivity and specificity of the images. Furthermore, it is necessary to acknowledge the temporal dynamics of PFA fixative penetration, inherent biological variability within a cohort of mice, and that our conclusions drawn from brain tissue may not universally apply to other tissues .

The fixation time should be carefully monitored and optimized to achieve the best possible fluorescence consistency. Fixation protocols should be made for PFA depending on the specific experiment design.

Acknowledgements

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