Heme Processing Pipeline: Correcting Non-uniform Illumination and Image Processing in Histology

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ABSTRACT

Heme, an immunomodulatory blood protein, has been shown to correlate with AD risk factors and affect the brain neuroimmune response, however, the mechanisms in which heme interacts with the neuroimmune system and affect AD pathology are poorly understood. Immunofluorescent histology is a technique commonly used to understand biological changes *in vivo* and, when used with a specialized heme sensor, can be used to investigate the effects of heme in the brain. The heme sensor has been successfully used with cell cultures but not histology because of two major image-processing limitations – (1) microscopic illumination aberrations prevent accurate analysis of the images and no tool exists to correct illumination aberrations in histology, and (2) a unique background correction and data normalization algorithm is required to analyze images produced from the heme sensor. Thus, we propose the Heme Processing Pipeline (HPP), a custom workflow in MATLAB and ImageJ that successfully overcomes these image-processing challenges and develops analyzable histology images from the heme sensor. An experimental set of images from wild-type mice are used to prove the efficacy of the HPP. The HPP will enable future *in vivo* studies using the heme sensor and help further elucidate the mechanisms of heme in the neuroimmune system and AD.

INTRODUCTION

Alzheimer's Disease (AD) is commonly characterized by senile plaques composed of amyloid beta and aggregated tau protein that are associated with neuronal cell death[1]. For decades, this approach has been the dominant framework, however, offers a rather narrow perspective. Recent research suggest that a multitude of supporting cells, immune cells, immunomodulatory factors contribute to broad dysregulation in an AD environment which collectively contribute to AD pathogenesis[2]**.**

Increased levels of heme, an immunomodulatory blood protein, is characteristic of AD. Heme is associated with a number of AD risk factors including age, brain injury, and stroke[3]. On a molecular level, heme has been show to co-localize and bind to amyloid-beta (AB) plaques, altering their aggregation state[4] and affect the neuroimmune response in macrophages and astrocytes[5]**.** Despite these initial findings, little is known about the effects of heme on aggregated protein, neuroinflammation, glial cells, and the mechanisms driving these interactions.

The effects of heme can be investigated using a ratiometric heme sensor combined with fluorescent microscopy. The heme sensor detects fluorescent intensity which is dependent on the amount of heme bound to a sample[6]. The heme sensor technique has been successful with cell cultures but not with histology because of multiple image-processing challenges. Fluorescent microscopy, especially in histology, is often affected by illumination aberrations. Microscope lenses can produce images with brighter intensities in the center and darker shading around the edges[7]. Uneven surfaces in the tissue, autofluorescence and background noise, and different anatomical structures can exacerbate these shading differences. Such difficulties prevent accurate accurate analysis of the image, and even more so when an image is tiled, resulting in aberrations on the border of each tile.

Computational tools exist to correct these aberrations, however, are **mostly geared towards illumination correction in cell cultures, not histology**. Current tools are not flexible enough to take into account the different shading aberrations created from tissue samples and thus have limited degrees of success. Additionally, images from the heme sensor **require unique background correction and data normalization steps** to extract signal. Thus, there is a need to develop an image-processing workflow that both integrates illumination correction and signal detection methods and is adaptable to varying microscopic aberrations.

This project developed the Heme Processing Pipeline (HPP), a computational workflow in MATLAB and ImageJ that processes tiled histology images from the heme sensor. The HPP successfully corrects non-uniform illumination aberrations using the ImageJ plug-in BaSiC[8] while removing background noise and normalizing signal to create analyzable images. The HPP is also an adaptable workflow, allowing individual image-processing steps to be turned on, off, and re-iterated. This computational pipeline will enable the analysis of future *in vivo* experiments using the heme sensor. It will also serve as a general optical image-processing tool for histology images, widening the possibilities of *in vivo* research.

METHODS

Heme Sensor

The heme sensor contains a heme-binding domain that is fused to the fluorescent proteins EGFP (excitation = 488nm) and mKATE2 (excitation = 555nm). Both proteins are expressed, however, EGFP is decreased upon heme binding while mKATE2 stays constant[6]. The heme sensor can use appropriate fluorescence channels to detect expression of each proteins. Images are taken with the heme sensor using the EGFP and mKATE2 channels, and the mKATE2 image can be used as a normalizing factor. Thus, processed images use EGFP fluorescence normalized to mKATE2 fluorescence to represent levels of bound heme.

Two different types of heme-binding domains are are used in this experiment. The first, HS1, contains a Methionine-Histidine region that is critical to successful binding[9, 10]**.** The second, BisAla, contains a modified heme-binding domain where the Histidine is replaced with an Alanine. This amino acid substitution interferes with the binding affinity. Thus, the heme sensor using HS1 is expected to strongly bind heme while the heme sensor using BisAla is expected to weakly bind heme.

Imaging

Four brain sections from wild type mice were used. A heme or vehicle solution is poured over each sample, and four images are taken:

1. image using the EGFP channel, with the heme-sensor off
2. image using the EGFP channel, with the heme-sensor on
3. image using the mKATE2 channel, with the heme-sensor off
4. image using the mKATE2 channel, with the heme-sensor on

The images with the heme-sensor off are used for background correction purposes. Images were taken at 20x magnification. The number of image tiles for vary to best fit the area of the tissue sample.

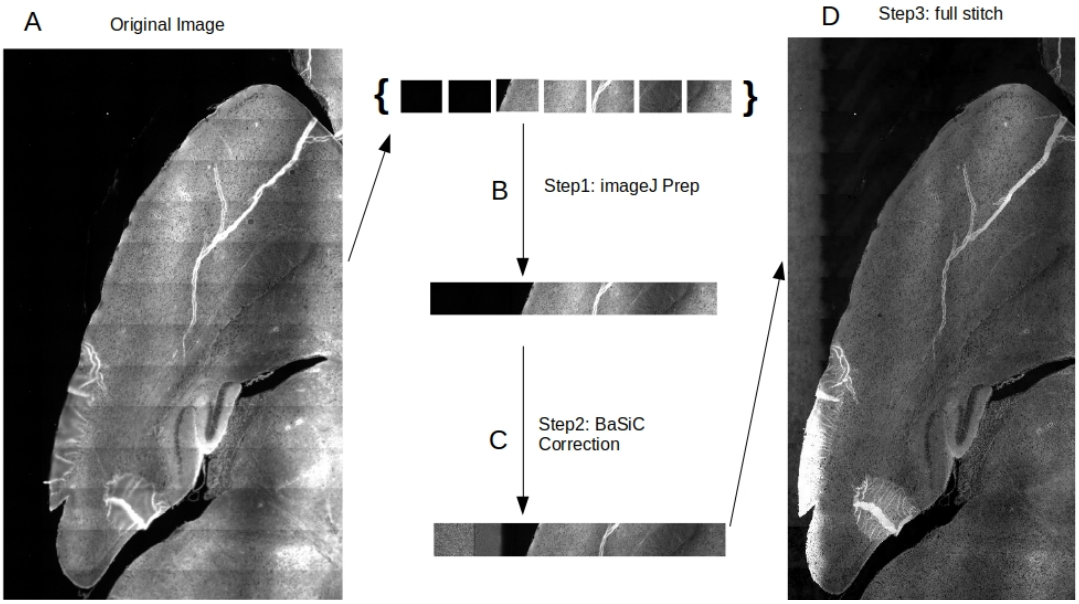
RESULTS

Heme Processing Pipeline

The HPP requires a specific folder hierarchy which can be set up using the program under Step0 and the README file.

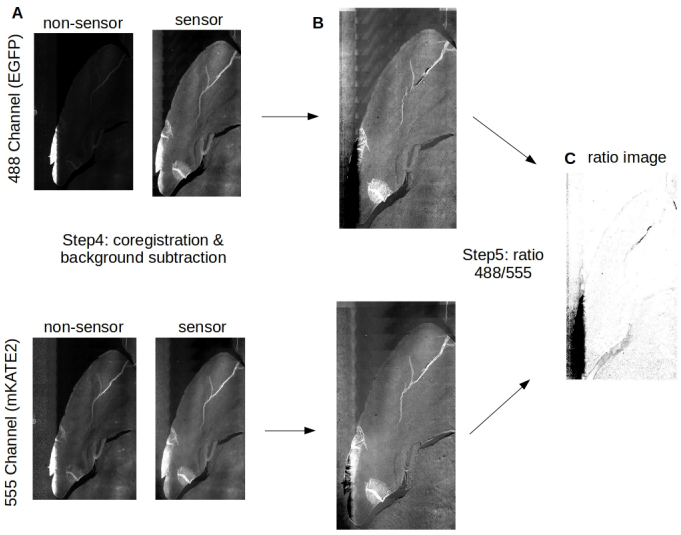
The HPP first corrects the non-uniform illumination within the tile images. In MATLAB, it stitches the image tiles horizontally, building a horizontal image strip for each row (Step1). The horizontal image strips are then saved. All the image strips are then collectively passed through BaSiC. BaSiC computes a dark-field and flat-field correction matrix which is applied to all horizontal image strips (Step2). The more horizontal image strips passed through BaSiC, the better the correction matrices. This step produces corrected horizontal image strips which are then saved. In MATLAB, the corrected horizontal image strips are stitched together to form a full image (Step3). This process is repeated for each image separately, and a unique dark-field and flat-field correction matrix is calculated for each image. Additionally, this image correction process can be iterated multiple times if the first iteration is not perfect. The BisAla 10uM sample was iterated twice since the first iteration did not generate a perfectly corrected image.

Figure 1. Correcting Non-uniform Illumination using BaSiC ImageJ Plugin. BisAla 0 uM Heme is used an example. (A) The image tiles stitched without correction. (B) Image tiles for a row is stitched together, building a horizontal image strip. (C) All horizontal image strips are passed through the BaSiC ImageJ plug-in. Corrected horizontal image strips are created. (D) The corrected horizontal image strips are stitched together.



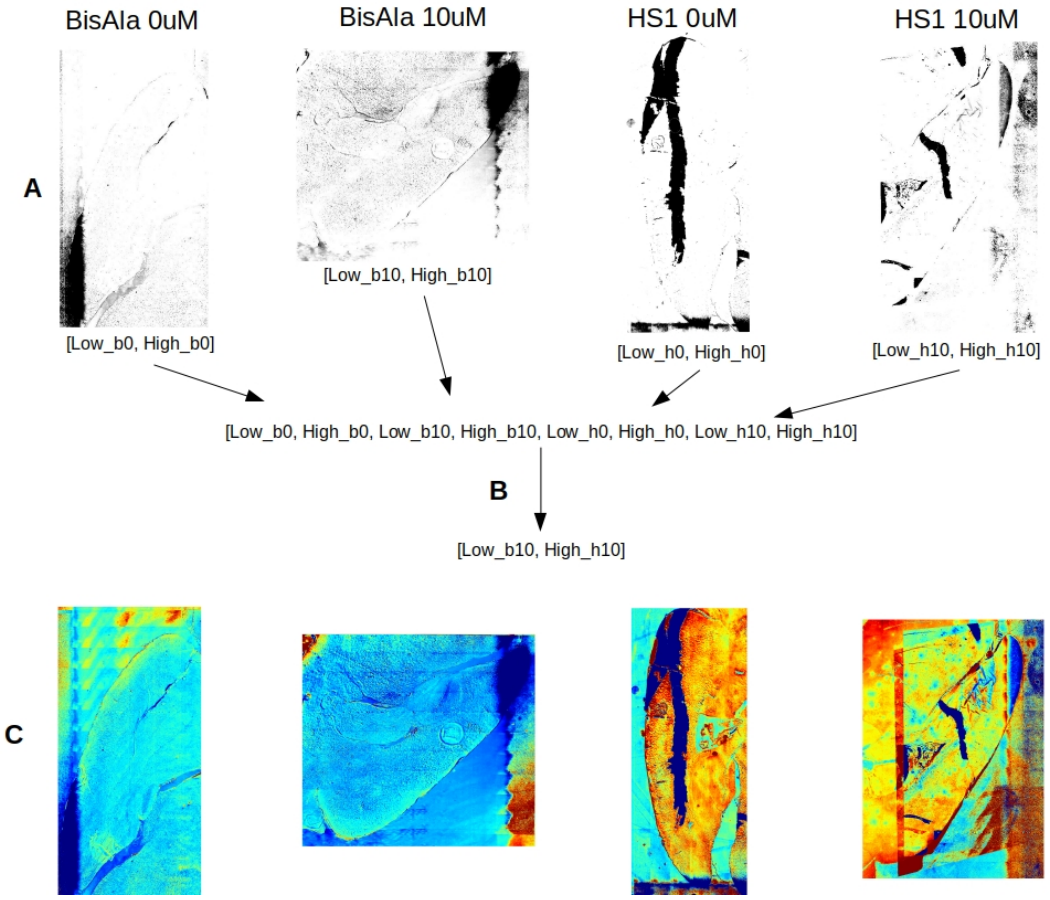
The HPP then applies background correction and normalization methods. Because the images do not have the same number of tile images and thus have different dimensions, a co-registration algorithm must be applied to ensure that the corresponding regions of the tissue from the non-sensor and sensor images are aligned. The MATLAB imregister function along with optimized parameters are used to co-register the images. Once the images are properly aligned, the non-sensor image is subtracted from the sensor image, pixel for pixel, to subtract background noise. This is completed for both the 488 (EGFP) and 555 (mKATE2) channel images. A ratio image, which represents normalized EGFP, is created by dividing the 488 image by the 555 image.

Figure 2. Co-registration and Background Subtraction to Build a Ratio Image. (A) co-registration of non-sensor and sensor images. (B) Background corrected image. (C) Ratio image consisting of 488 (EGFP) channel image divided by the 555 (mKATE2) channel image.



The HPP then computes a universal color map for the ratio images. MATLAB generates a custom color map for each image, however a universal color map allows for comparison between images. A universal color map must have a range that encompasses the pixel values across all images, then maps each value within that range to a color. For each ratio image, the highest and lowest pixel values are determined, and then further sorted to find the highest of the highs and lowest of lows. These two values are ensured to capture every pixel value across all ratio images. Images, however, can have outlier pixel values, thus creating an excessively wide color range. An excessively wide range will skew the color shading such that the majority of the pixel values correspond to a small spectrum of colors. Thus, the HPP has a built in method to manually set the range for the universal color map.

Figure 3. Determining a Universal Color Map. (A) The highest and lowest pixel value for each ratio image. (B) These values are combined creating a single array of all the highest and lowest values. The highest and lowest values are determined. (C) A single color range is applied to all ratio images.



The code and a demo can be found on the author's github:
https://github.com/bioben/heme_image_processing.git

DISCUSSION

The HPP successfully processes histology images from the heme-sensor. It successfully corrects non-uniform microscopic illumination and applies background and normalization corrections to create analyzable images. It is an adaptable tool that allows image-processing steps to be turned on, off, or re-iterated which allows for a more refined method to process histology images.

The HPP effectively corrects non-uniform illumination while producing images that preserve shading and morphological details. Tissue samples are nonhomogenous and contain multiple sources of

variability such as imperfect binding and different anatomical regions across a tissue sample. These differences create variance in fluorescence and must be preserved throughout the image processing steps to accurately analyze these images. However, other sources of variability such as background autofluorescence and microscopic aberrations must be removed. Current image correction tools[11, 12] remove all shading details and fail to preserve these biological details. These correction tools are mostly geared towards cell culture images where the variability in fluorescence is not as a large. The HPP successfully removes microscopic aberrations while retaining biological shading and morphological details. In the final images, tiles are stitched together seamlessly while splotches and biological shading are preserved. Blood vessels, uneven tissue, and brain regions are still clearly defined. More validation experiments should be conducted to prove the efficacy of the HPP on histology images from different microscopes, however, the results demonstrate that the HPP is successful in being a correction tool for histology images.

The HPP modifies its use of BaSiC, an ImageJ plug-in that corrects non-uniform microscopic illumination. BaSiC takes a set of individual tile images, determines a correction matrix, and applies it, creating a new set of corrected tile images. Instead of passing each individual tile, the HPP passes horizontal image strips through BaSiC. When individual tile images are used, BaSiC fails to remove the illumination aberrations. The author is unsure as to why this occurs and why horizontal strips successfully remove the illumination aberrations. More work should be done to understand the mathematical concepts behind BaSiC.

The HPP is programmed in a step-wise manner such that it can be easily adapted to different image processing needs and experiments. Steps such as the non-uniform illumination correction can be iterated multiple times if one iteration is not sufficient to correct the illumination aberrations. This is incredibly useful and makes the HPP a successful tool in correcting illumination aberrations in histology since histology images can contain large amounts of variability. For example, two iterations of the illumination correction step were applied to the BisAla 10uM sample while the other samples only required one. Steps can be by-passed, rearranged, or used individually. For example, the non-uniform illumination correction can be isolated and used for any microscopic image containing the same aberrations. The universal color map algorithm can also be isolated and used for any set of images. In this sense, the HPP is a general tool for any histology or microscopic.

One drawback is that the HPP is not fully automated. In this experiment, each image contained a different number of tile images and thus was a different size and dimension. These components were hard coded into the HPP. In future experiments, images should be the same size. Additionally, the HPP should be modified such that it reads the CZI file instead of exported tile images. Using the CZI file has multiple advantages such as easy access to the image metadata including its dimensions and reduction of file clutter that comes with individually exported tile images. MATLAB and ImageJ are both capable of reading CZI files and thus CZI-compatibility is a feasible next step for the HPP.

The HPP will enable future *in vivo* experiments investigating the role of heme in Alzheimer's Disease. Images taken using the sensor can be effectively and efficiently processed such that meaningful data, for example heme-AB co-localization, heme binding efficiency, and heme aggregation, can be analyzed. Experiments using wild type and AD-phenotype mice models can be used to further understand the effects of heme *in vivo*. Additionally, the algorithms from the HPP can serve as an additional tool to solve a variety of common image-processing challenges.

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