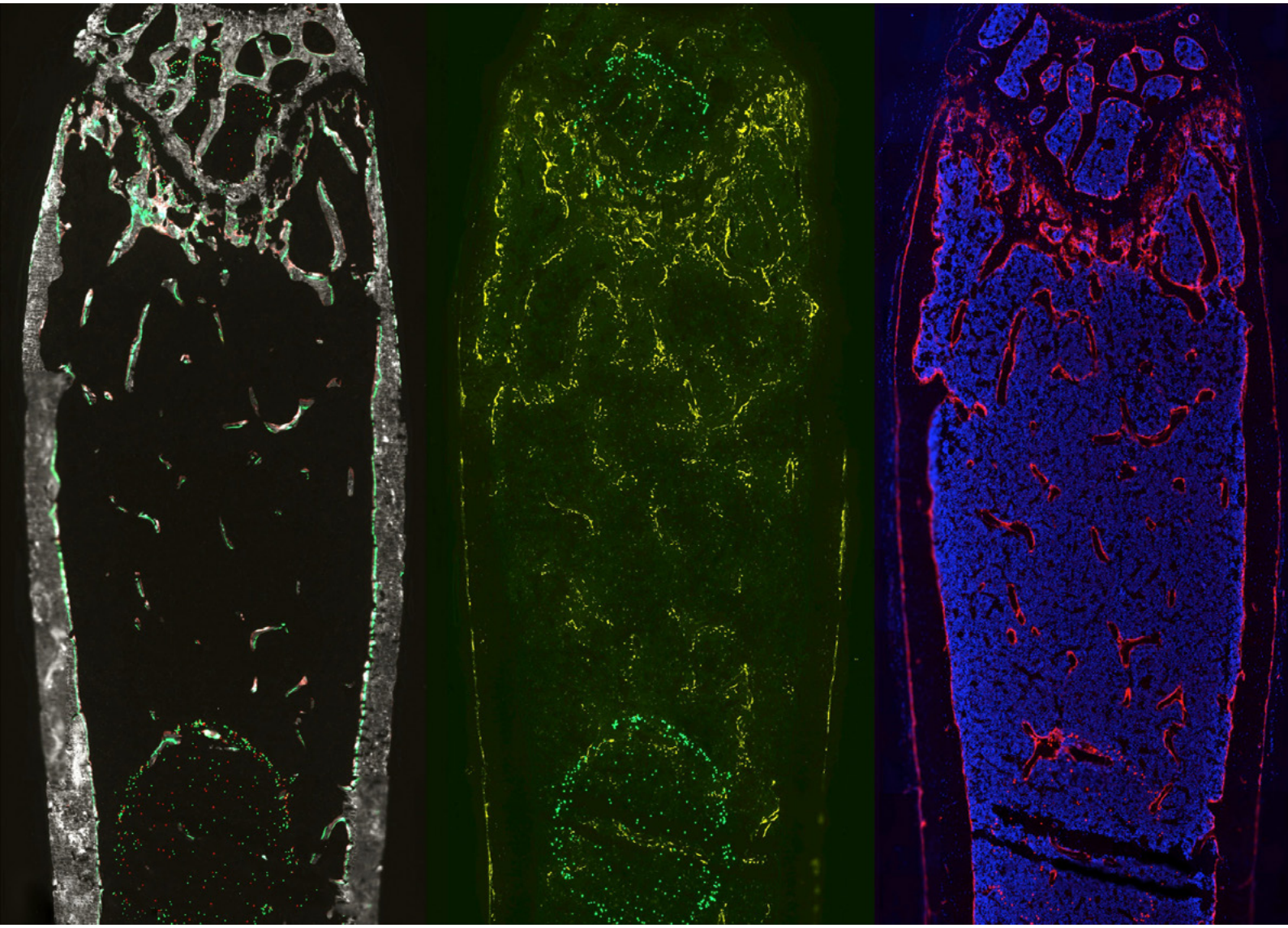

ZEISS Axio Scan.Z1

High Throughput Cryohistology of Mineralized Tissue

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High Throughput Cryohistology of Mineralized Tissue

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Introduction

The fundamental contribution of histology to the field of genomics is the ability to associate a specific molecular / chemical signal to a regional domain or cell type. For example, demonstrating that an expressed RNA obtained from a whole tissue extract is localized to one cell type and the encoded protein is also present either within or around these cells provides the needed detail for interpreting an RNA expression study. Histological methods utilizing a variety of molecular probes exist to record these responses in tissue section but the integration of multiple measures to a specific cell or region is difficult.

Despite the rich and expanding source of probes for interrogating a tissue sample, the traditional histological tools for discriminating tissue heterogeneity are still primarily based on chromogenic chemical stains, which can mask the activity of some probes. A two-step process of biological probe followed by the chromogenic stain is used to localize the position of the probe signal to the histological section. However, multiple sequential probing steps need to be performed on adjacent tissue sections with the expectation that both probes will align with the closely related morphological features. These steps are labor intensive and are not readily

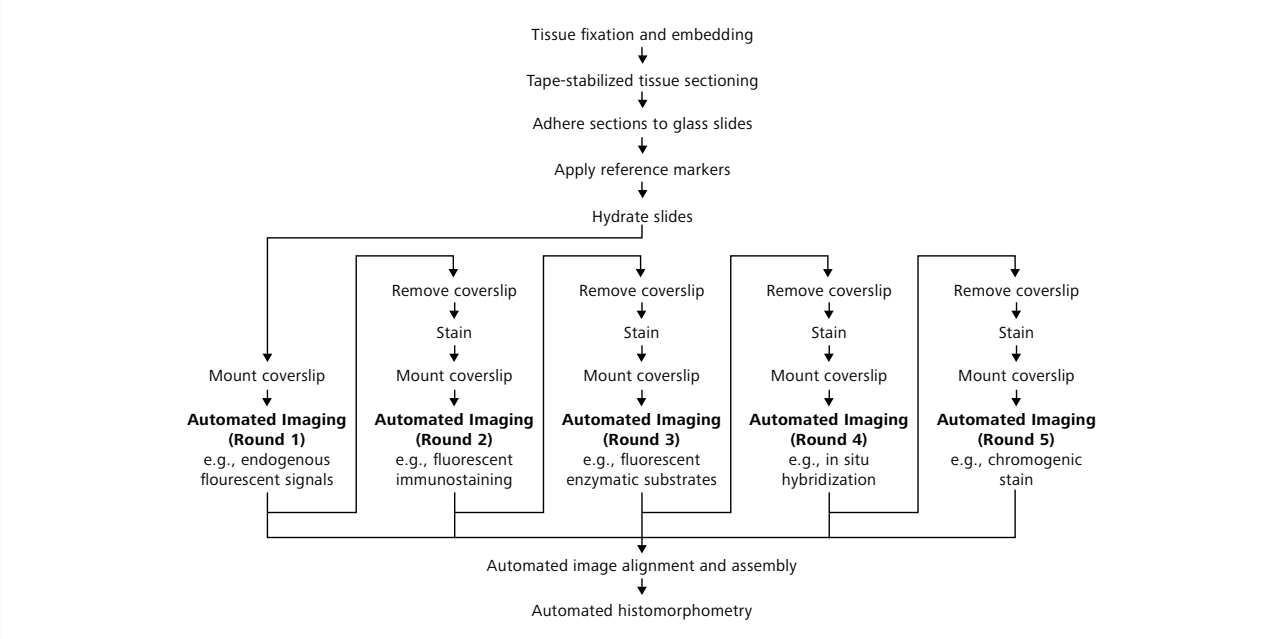

Figure 1 *Workflow for high throughput multiple image cryohistology.*

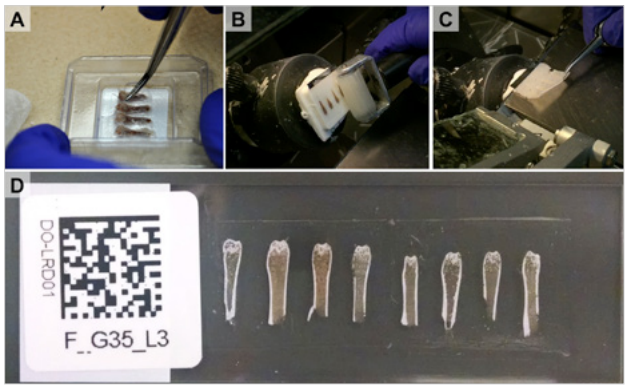

Figure 2 *Example steps of the cryohistology preparation protocol. (A) Positioning and aligning mouse femurs in a cryomold with OCT prior to freezing. (B) Applying the Cryofilm to the tissue block with a hand roller. (C) Cutting the mineralized section while guiding the Cryofilm free from the block face. (D) Sectioned mouse femurs collected on two pieces of Cryofilm tape adhered to a microscope slide.*

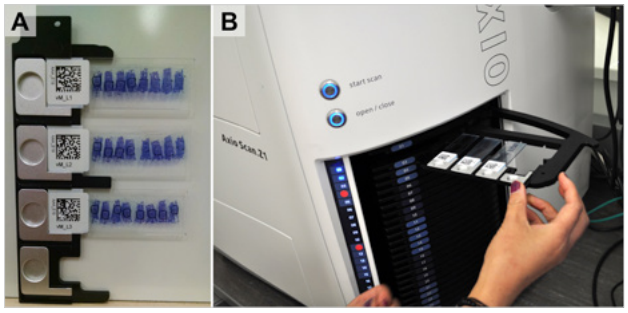

Figure 3 *(A) Multiple slides in the slide tray of Axio Scan.Z1. Slides are held rigidly in a tray facilitating the alignment of images from multiple rounds of staining / imaging. (B) Loading the slide tray into the tray stack of Axio Scan.Z1.*

adaptable to image analysis because of the difficulty of aligning the probe signal obtained from multiple tissue slices. Some improvements in extracting multiple signals from a section has been achieved with both high content analysis of wide field and confocal scanning microscopes, but the limitation of repeated probing and imaging of the same section remains a significant technical hurdle. Recently, we published an investigation of the development of tendon attachment to bone in young mice using repeated rounds of staining and imaging of a single tissue section[1]. We also utilize these methods in addition to automated dynamic histomorphometry measurements to phenotype the skeleton of mice[2]. In the following pages, we outline the strategy used in greater detail.

Multiple Image Cryohistology Workflow

An overview of the workflow for high throughput multiple image cryohistology can be seen in figure 1. Tissues can be mounted on a slide after sectioning and a single section of tissue can be stained and imaged in multiple rounds. Using ZEISS Axio Scan.Z1. images from each round are aligned computationally and analyzed. This method is preferred to labeling of serial sections and allows for alignment of a combination of fluorescent and chromogenic labels for highly accurate spatial information.

1. Fixation and high-throughput embedding

Tissues can be fresh-frozen or fixed in formaldehyde prior to embedding. When applicable, multiple samples can be embedded in parallel to increase sectioning throughput (Figure 2A).

2. Tape-stabilized tissue sectioning

Dr. Tadafumi Kawamoto (www.section-lab.jp) developed a tape stabilization strategy that uses adhesive coated polyvinylidene chloride film (Asahikasei Kogyo, Japan) to capture the tissue section[3]. In their protocol, a fresh frozen tissue is sectioned onto the tape and immediately fixed in PFA or ethanol. Subsequently, the tape is mounted on a glass slide with the sample side down for microscopic examination. Sequentially cut sections are placed on separate slides and different staining protocols may then be performed on each section of tissue.

We have modified and added to the Kawamoto protocol in a number of ways including fixing the sample in PFA prior to embedding. This step is necessary to fix soluble cytoplasmic GFPs within the limits of the cell. We have utilized the Section-Lab tape (Cryofilm Type-2C) for a wide range of tissue types. Laboratory personnel with limited histological experience can produce high-quality sections with this method. The primary advantages of this protocol are the relative low cost (no special instrumentation, < $1.00 per slide), no loss of mineral fragments as seen with other tape transfer methods and the ability to perform multiple rounds of staining and imaging on the same section (Figure 2B and 2C).

3. Adhesion of sections to microscope slides

The captured section is adhered to the glass slide (tissue side up) using either chitosan adhesive or UV activated glue (Figure 2D). With the unstained section now facing upward,

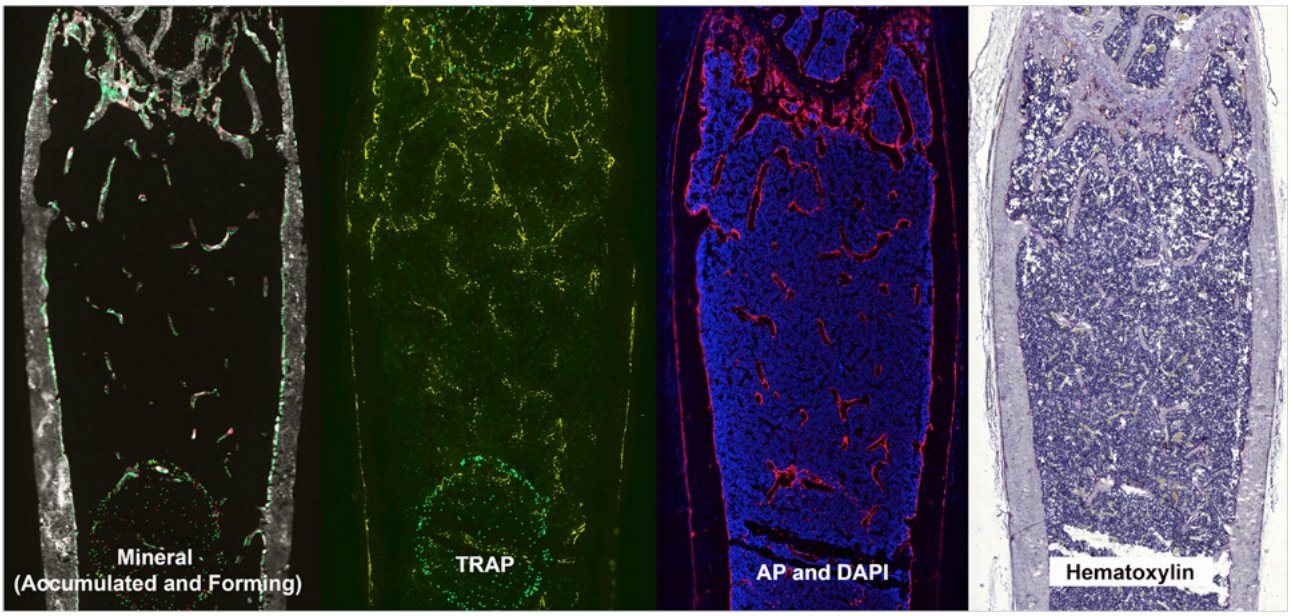

Figure 4 *A single bone section processed through four rounds of staining and imaging. From left to right, (A) mineralized tissue containing mineral labels (green, red) and stained with calcein blue (white), (B) tartrate resistant acid phosphatase (TRAP) stain for osteoclasts (yellow), (C) alkaline phosphatase (red) and DAPI nuclei (blue), and (D) hematoxylin stain. These images are exported as image layers that can be used for subsequent image analysis steps.*

a removable cover slip (30 % glycerol) is placed over the section for microscopic examination. Subsequent staining steps begin by floating the coverslip off by submerging the slides in liquid (e.g., 1X PBS or H_2O) and performing the staining protocol on the tissue section while still adherent to the glass slide.

4. Application of reference markers to slides

Green and red fluorescent microsphere calibration solutions are mixed and applied near the periphery of the tissue sections after the adhesive has dried. These microspheres are acquired during each round of imaging to provide a reference for alignment in step 7.

5. Multiple rounds of staining

By choosing a compatible sequence of imaging, staining, and reimaging steps, it is possible to detect and co-localize many biological signals on the same tissue section. Each round of imaging/staining/reimaging has to be developed for the particular histological question. The imaging / staining / reimaging sequence typically involves acquiring the endogenous fluorescent signals (e.g., cellular GFP, mineralization dyes, in vivo imaging probes) on the first round of imaging followed by fluorescent multiplexed immunostaining, enzymatic activity stains, and even in situ hybridization. Lastly, the section can be stained using chromogenic dyes (e.g., H&E, toluidine blue, safranin O, etc.) to highlight the tissue architecture.

6. Multiple rounds of imaging with ZEISS Axio Scan.Z1

Experiment profiles designed for each fluorescent or chromogenic label are used to identify the sample tissue and collect image data at each imaging step. After imaging, the slides are removed, processed for the next staining step, reinserted into the microscope, and scanned again for the next labels. The slide tray of Axio Scan.Z1 holds the slides in a reproducible manner, facilitating multiple rounds of imaging of a section of tissue (Figure 3). Individual channels of a single image collected from each step are collected with the color or black and white cameras of Axio Scan.Z1 (Figure 4). For each round of imaging, the individual tiled images are automatically labeled with a predefined naming protocol (sample ID, imaging condition) that is subsequently used in the image processing step.

7. Image assembly and analysis

A four stage process is applied to the files associated with each tissue section (Figure 5). First, the images are stacked and vertically aligned using the fluorescent calibration microspheres that are included in each image. The process corrects for minor rotational errors that occur between each imaging round as well as subtle misalignment errors caused by minor expansion / contraction movements of the tape. This is a particularly computer intensive step which is facilitated by using a computer cluster configuration and

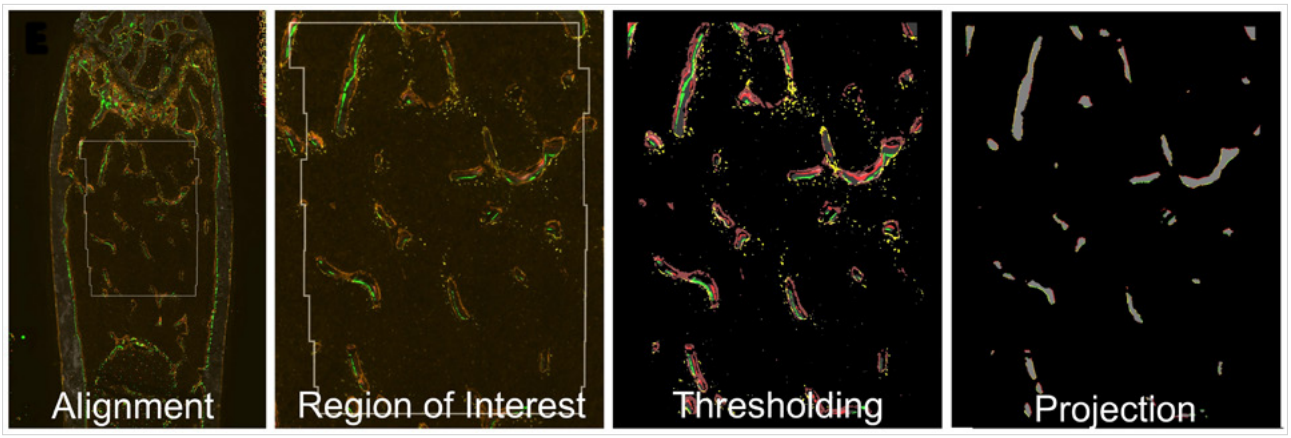

Figure 5 *The four major steps that are performed in the image analysis program. The alignment step is dependent on the registration beads that are embedded in each sample. The process corrects for minor degrees of rotation and expansion-contraction of the tape during the enzymatic steps. The region of interest requires that defects within cortical bone have to be identified to maintain the surface of the cortical bone. Once the cortical bone is identified, then the ROI can be determined. The thresholding step corrects for background intensity and uses algorithms to distinguish the presence or absence of each signal collected. The result is a binary map of each signal relative to the trabecular bone. Projection moves each signal back to the surface of the bone and is used to measure the percentage of the bone surface that contains the signal.*

explains one of the reasons for performing the task in a cloud-based format. Once completed, the stacked image can be viewed on the computer using a layered viewing program (e.g., Photoshop, GIMP, etc.) with the ability to turn on or off any combination of layers.

The analysis (second) phase is initiated by thresholding each fluorescent signal to a binary value. Each signal is then mapped to the tissue registration image (mineral, DIC, chromogenic) in which the region can be defined by unique computer-defined image rules. In stage three, a region of interest (ROI) is determined by computer-defined rules. Then within the ROI, the computer associates the multiple signals to the registration image and using unique biological relationships between the signals, produces a quantitative analysis of the image. Finally, the projection phase of the analysis compares the overlapping signals from each image layer and calculates the percentage of bone surface containing each signal from each tissue label.

Discussion

Here we have presented a detailed cryohistology protocol to quantify and co-localize several biological measures by aligning images from multiple rounds of staining / imaging on a single section. The method outlined using Cryofilm tape is especially useful as it maintains the morphology of difficult to section tissue (e.g., mineralized bone). In addition, the sectioned tissue is adhered firmly to the glass slide, allowing for multiple rounds of staining / imaging of the same section; unlike traditional methods where serial sections are each stained with a different protocol. Using serial sections can pose an issue when attempting to co-localize signals between the sections, something that is not a problem with single sections that have been stained and imaged with multiple rounds. This protocol represents an advancement in high throughput cryohistology and should be of use to those imaging difficult to section tissues such as bone as well as those inexperienced with tissue sectioning.

References:

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