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General Introduction

Histology is the study of the microscopic anatomy and cytoarchitecture of tissues. Pathology is the study of diseases, especially in the context of their structural and functional alterations. Histopathology is the branch of pathology that deals with the examination and characterization of diseased cells and tissues.

Good histopathology studies are dependent on a linear sequence of events: **Collection – Fixation – Trimming – Embedding – Sectioning – Staining – Visualization – Interpretation**. Though there are situations in which some of the steps may be omitted, each step is dependent on the previous one and is defined as well as restricted by both the **stage of progression** and the **desired result**.

Detailed knowledge of the performance of all previous steps in the sequence is **crucial** to obtaining the best possible result.

Clear understanding and definition of the expected result is **necessary** for choosing the correct parameters for each step of the process.

Collection

In the Histopathology Facility, we process several types of samples including whole organisms, isolated organs, slices of tissues or groups of tissues, cell cultures (both 2D and 3D), fluids and even inorganic materials.

Each sample type requires its own special considerations and specific collection techniques. Additional parameters may be necessary depending on the downstream processing steps and desired outcomes. Therefore, it is always beneficial to **contact us before starting the collection process** to discuss potential requirements and optimal protocols.

For all biological specimens, the **time between collection and preservation** has immense impact on the quality of the results. Once autolysis has set in, no amount of processing can undo it.

Things to consider

1) When collecting samples for histological analysis, avoid artifacts such as tearing, breaking, squishing or drying of the sample.

2) Clearly label all collected samples with **a unique identifier** (e.g. mouse ID or organoid batch number), other relevant details (such as genotype, treatment or other procedure), **submitter initials and date**. The clearer and more complete the information provided, the lower the risks of downstream errors.

3) Ensure that there is no room for sample misidentification (e.g. between samples of multiple groups or for multiple samples submitted together in one container)

Crucial parameters

Maximal size – no restrictions provided the sample is placed in a proportionately sized container that allows adequate immersion in the fixative

Minimal size – if it is possible to manipulate the sample manually or using a pipette it is appropriate (down to around 200 µm in the smallest dimension)

Time from collection to fixation – not longer than 30 minutes provided the sample is kept in a cool place and away from direct sunlight

Fixation

Fixation is the physical or chemical process which **preserves biological samples** and **prevents their degradation** by bacteria and lytic enzymes.

Fixation techniques can inactivate many fluorescent proteins and enzymes in the sample. If observation of native enzymatic activity or fluorescence is needed, samples can be preserved by freezing rather than by chemical fixation.

There are many fixatives and fixation protocols that can be used, and their selection is mostly dependent on their compatibility with downstream processes.

All commonly used fixatives are a health hazard and require the use of personal protective equipment.

Things to consider

For proper fixation the sample needs to be accessible to an adequate volume of fixative of appropriate concentration for the time required for the fixative to properly penetrate through. Therefore, factors like: **ratio of the fixative volume to the sample volume**, **sample thickness**, **presence of barriers interfering with permeabilization** and **duration of fixation** need to be considered.

Crucial parameters

Duration of fixation – depends on the type of fixative and the type of sample. Cells and cell monolayers need 5 – 30 minutes; tissues usually need 16-18 h (typically not more than 48 h)

Type of fixative – mostly formaldehyde based (e.g. 4% paraformaldehyde, 10% Neutral Buffered Formalin, Bouin's Fixative)

Volume of fixative – not less than 20x total tissue volume

Trimming

Trimming **enables proper orientation** of the region of interest as well as **optimal sample size** for proper fixation.

On the other hand, it **can introduce physical artifacts**, such as tears or squish artifacts. Sometimes incorrect trimming can also lead to improper tissue orientation or loss of the regions of interest.

Things to consider

1) Always keep the appropriate / desired result in mind.

2) Plan ahead and mark all the necessary reference points.

3) Always trim before processing. Otherwise the tissue will be brittle and impossible to trim without introducing several artifacts.

Crucial parameters

Size of the tissue – usually not bigger than 3cm x 2cm and 3mm thick

Embedding

In the Histopathology Facility two types of embedding are routinely used: **paraffin embedding** and Histopathology Facility.

Paraffin embedding – sample is fully dehydrated and infiltrated with paraffin

Cryo-embedding – sample is frozen with or without the presence of cryoprotectant (30% sucrose)

Paraffin embedding	**Cryo embedding**
Provides better preservation of tissue architecture	Typically results in lower tissue quality due to some artefactual distortion of tissue architecture
Allows for longer, less problematic storage	Requires a -80°C freezer for long term storage
Results in loss of enzymatic or fluorescent activity in the tissue	Retains most of the enzymatic or fluorescent activity of the tissue
Allows for sections down to 1um of thickness	Usually sections down to 5 µm can be obtained
Paraffin embedded samples do not present any biological hazard	Cryo-blocks may present biological hazard
Immunostainings generally require additional antigen retrieval steps	Immunostainings require fewer antigen retrieval steps

Sectioning

Equipment for sectioning is chosen depending on the **embedding technique** and the **desired thickness** of the section. All sectioning devices in the Histopathology Facility can be booked and used independently **after an introduction** to the respective machine by a member of the Facility.

Machine	**Typical sectioning thickness**	**Embedding**
Microtome	1-6 µm	Paraffin embedding
Cryostat	5-20 µm	Cryo embedding
Sledge Microtome	30-150 µm	No embedding – frozen tissue
Vibratome	50-800 µm	No embedding

Sections are collected either by **flattening out** on slides (typically glass) or as **free-floating slices** in a solution. The latter is more common for sections from a vibratome. Appropriate technique of mounting the sections on the slides is necessary for an artifact-free result. After being mounted, the sections need to dry before they can be stained.

Things to consider

1) Typical artifacts created during sectioning include: tissue that is torn or ripped apart, wrinkles, folds, stripes, sections of uneven thickness and air bubbles. For good staining, interpretation and imaging, the number of artifacts on a slide should be minimized by handling the tissue as carefully as possible, changing blades when dull, and sectioning in a steady motion.
2) For harder tissues, like bones (decalcified after fixation) special blades are available and should be used.
3) Incomplete drying of the section on the slide might result in loss of tissue or partial staining.
4) Many problems of sectioning are often a consequence of improper fixation or embedding.

Crucial parameters

Section thickness – needs to be chosen depending on the staining and visualization modalities. Thinner sections have lighter staining patterns, but better cellular resolution

Section localization on the slide – number of sections per slide and their placement on the slide dependent on the downstream procedures. Generally, it is good to avoid placing sections close to the borders of the slide, as they are difficult to stain evenly and observe microscopically.

Staining

The two main types of staining offered by the Histopathology Facility are histochemical and immunohistochemical. Histochemistry utilizes techniques used for the visualization of various chemical components in tissues and immunohistochemistry is based on the selective, reversible and non-covalent binding of antigens by antibodies.

Good staining results are **strongly dependent on all the previous processing steps**, as changes in chemical properties of the tissue, enzymatic activity or availability of antigens can significantly impact the ensuing process and its outcome. Therefore, it is **crucial** to understand the principles and requirements of the intended staining and to prepare the material accordingly. If multiple staining procedures are intended on the same tissue section, **sequential compatibility** of the steps and reagents should be considered.

Visualization and Interpretation

The aforementioned processing steps performed in the Histopathology Facility typically culminate in a set of slides with one or more stains. Subsequently these slides are visualized, interpreted and imaged as required. The following aspects are worth discussing in the context of visualization and interpretation.

Quality control assessment

When pertinent, the slides are reviewed by light microscopy and the staining patterns in the submitted samples are assessed in tandem with reference positive and negative control slides. This ensures that the staining outcomes are adequate and valid. Usually such an assessment is conducted by the pathologist and the Histopathology Facility member who performed the staining. In some instances, this is done by other specialists or the users themselves.

Microscopic Examination

The slides are typically examined by brightfield or fluorescence light microscopy depending on the type of stain. In other instances, slides (usually stained by immunofluorescence) are examined by confocal or other microscopic modalities. On the Vienna Biocenter Campus, microscopic examination is typically performed by users at the IMP or MFPL Biooptics core facilities or within individual laboratories. Specific details pertaining to each microscope and microscopy method are available through the respective Biooptics facilities.

Whole slide digitization

Whole slide digitization, more commonly known as slide scanning, refers to the acquisition of a digital file featuring the visible information on a glass slide using specialized hardware that is referred to as a scanner. Such a file, called a scan or digital slide, can be archived and retrieved electronically. This enables the performance of "virtual microscopy" and quantitative analyses using specialized software. On the Vienna Biocenter Campus, whole slide digitization is offered as a service by the IMP Biooptics core facility (for users from IMP, IMBA and GMI). When appropriate, members of the Histopathology Facility work collaboratively with Biooptics staff to facilitate slide scanning. Specific details regarding instrumentation and digitization procedures are available through the Biooptics facility.

Things to consider

1) A good digital slide depends on a good glass slide which in turn depends on proper processing steps.
2) Staining intensities might need additional optimization for digitization of certain tissues and stains.
3) In some instances, the color profiles of tissues might appear different between light microscopic images and corresponding digital (virtual microscopic) images.

Imaging

A well-prepared slide with a good result is only as appreciable as the image that finally represents it. Images can be prepared by micrography (microscope and camera) or virtual microscopy (scan and computer). Specific details are available from the Biooptics facility. It is worth briefly emphasizing that the cytologic/histologic features of interest and meaningful staining patterns should be adequately and faithfully represented with minimal artefacts.

Comparative Pathology Services

A comparative pathologist is a medical or veterinary professional with specialty training in the discipline of pathology, expertise in the biology and pathology of model laboratory animals, and familiarity with the comparative features of human diseases.

In many projects, comparative pathology support is beneficial for the morphologic evaluation and correlative interpretation of tissue phenotypes in model organisms. The histopathology facility features in-house comparative pathology expertise to provide the following services:

- Project planning support and advice on experimental design
- Consultative reviews of staining outcomes
- Microscopic analyses of morphologic features and representative imaging
- Digital quantification (in collaboration with IMP Biooptics)
- Correlative interpretation and validation of histomorphologic findings in relation to findings from other modalities

General considerations

1) Plan ahead to choose the microscopic modality that is necessary and appropriate. Then, arrange with the Histopathology Facility to prepare the tissues accordingly.
2) Avoid visualization modality mismatches (inadequate or excessive for the specific sample and its relevant features) and consequent under- or over- interpretation.
3) Perform visualization and interpretation with the requisite frames of reference and appropriate positive and negative controls.
4) Consult with a pathologist or other specialist when pertinent. Analytical errors (including errors of histological interpretation) are a notable cause of retraction in the scientific literature. (*FASEB J*. 2014; 28(9): 3847-3855)

"*Report what you see and not what you want to see*" is a common saying among pathologists. Sometimes it is important to thoroughly examine and accurately interpret the microscopic features and staining patterns in the tissue without being disproportionately influenced by findings in previous reports and features in other models.