Histology Revolution: From Inefficient, Two-Dimensional, and Low-Resolution Techniques to High-Throughput, Three-Dimensional and High-Resolution Techniques

Review Article

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ABSTRACT

Histology has been the gold standard for studying the cellular organization of body tissues and organs for several decades. The common procedures for histology studies generally include fresh tissue acquisition, tissue fixation, processing (for paraffin sectioning), embedding, sectioning, staining, and imaging. Various fixative methods, embedding materials, and sectioning methods have been developed to achieve different staining purpose. There are many histological staining methods used to study tissue characteristics and microscopic structures of tissue: including Immunohistochemistry (IHC) / Immunocytochemistry (ICC) / Immunofluorescence (IF) for detecting specific proteins, in situ hybridization (ISH) for specific DNA and RNA, Sudan staining for lipids, Sirius Red staining for collagens, routine Hematoxylin and Eosin (H&E) staining, and others. However, these techniques have some limitations: inefficient, two-dimensional, and low-resolution. Accordingly, scientists are putting efforts into developing more efficient, accurate, and high throughput histological techniques. Recently, several new histological techniques have been devised. These new techniques include the cytoplasm-specific / nucleus-specific X-ray staining for three-dimensional (3-D) histology of soft-tissue samples, the tissue clearing techniques (OPTIClear), and advanced in situ sequencing techniques: fluorescence in situ sequencing (FISSEQ), spatially resolved transcript amplicon read-out mapping (STARmap), and Slide-seq. These new techniques will significantly facilitate histological and histopathological research including identification of stem-cell inches, genome-wide spatial gene expression, tissue organization and functions. However, these new techniques also have some drawbacks: such as high background and generation of a plethora of data. Here, we summarize the most commonly used techniques and the latest advanced histological techniques, as well as their advantages and disadvantages.

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INTRODUCTION

Histology is the study of cellular structure of tissues and organs. There are various histological methods developed for detecting class-specific molecules, such as Immunohistochemistry (IHC)/ Immunocytochemistry (ICC)/ Immunofluorescence (IF) for proteins, in situ hybridization (ISH) for DNA and RNA, Sudan staining for lipids, Sirius Red staining for collagens and others[1-4]. However, these traditional histological stains have some drawbacks: time-consuming, two-dimensional images, inefficient, and low-resolution. Accordingly, the next generation of histology calls for highly efficient, accurate, high-throughput, high-resolution, and 3-D histological techniques. Recently, scientists devised several new histological techniques: cytoplasm-specific/ Nucleus-specific X-ray staining for three-dimensional (3-D) histology of soft-tissue samples[5,6], tissue clearing techniques (OPTIClear)[7-11], and advanced in situ sequencing techniques including fluorescence in situ sequencing (FISSEQ)[12], spatially resolved transcript amplicon read-out mapping (STARmap)[13], spatial transcriptomics[14], and Slide-seq[15]. This allows scientist to investigate the precise cellular structure of tissues, draw detailed 3-D maps of tissues, measure genome-wide expression at a cellular resolution, or test where particular genes are active in a tissue, an organ, or even whole organisms. In this review, we provide a general introduction of the traditional histological techniques and recently developed advanced histological techniques.

Traditional histological techniques

The procedures for histological staining generally include fresh tissue acquisition, tissue fixation, processing (for paraffin sectioning), embedding, sectioning,

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staining, and imaging[1,16]. Fixation is a critical step in the preparation of histological samples, as it can preserve the tissue structures and stop autolysis and putrefaction. There are two main tissue fixation types: chemical fixation and physical fixation[1]. Chemical fixation is divided into several different groups according to the fixative solution, including aldehyde fixation, oxidizing agent fixation, alcohol fixation, mercurial fixation, and picrate fixation[1,4,17]. Physical fixation includes freezing and micro-waving[1].

After fixation, in most cases, the tissue needs to be immersed in a solid material to support the tissue for sectioning. The most commonly used supporting materials are paraffin wax, O.C.T., resin[1,4], and TFM Tissue Freezing Medium[4]. For paraffin and resin embedding, specimens need to be dehydrated and cleared through processing, which better preserves structure morphology but may lead to antigen masking and loss of hydrophobic components[1]. On the other hand, O.C.T. and TFM Tissue Freezing Medium are usually used for freezing tissues[4].

Once embedded, the specimen is sectioned or cut into thin slices using a special cutting tool such as a microtome, heavy-duty microtome, cryotome, vibratome, or ultramicrotome[1]. The choice of the embedding material and the sectioning method is mainly dependent on the fixative methods and the staining after sectioning. Table 1 lists the most commonly used fixation methods, embedding materials, sectioning methods, and suitable stains (Table1).

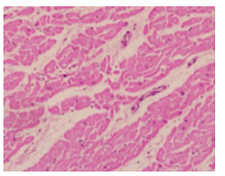
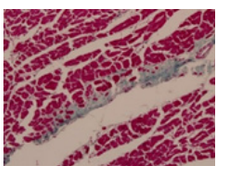
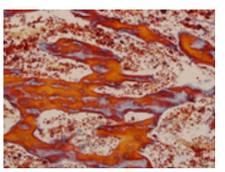
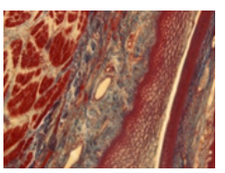
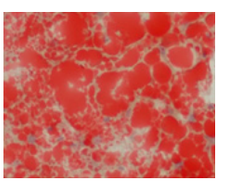
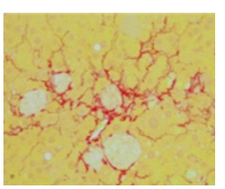
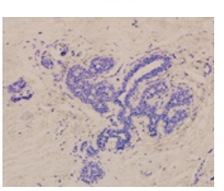
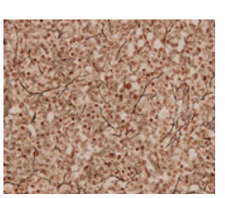
Table 1: Most commonly used fixation methods, embedding materials, sectioning methods

Machines for sectioning	Embed-ding material	Commonly used fixative method	Typical section thickness	Histological staining
microtome	paraffin wax	Formaldehyde	3-10 μm	H & E staining[1], IHC/IF staining[1]. In situ hybridization[3]
cryotome	OCT/TMF	Fresh freezing	3-10 μm	In situ hybridization (RNAscope)[2], Slide-seq[15], lipid staining[1]
		4% PFA freezing	3-10 μm	Lipid staining[1], In situ hybridization[18], IHC/IF[1,4].
vibratome		Fresh freezing	10-30 μm	In situ hybridization[2], Slide-seq[15], lipid staining[1]
		4% PFA freezing	100-300 μm	Lipid staining[1], In situ hybridization[18], IHC/IF staining[1].
ultramicro-tome	Resin	2% glutaraldehyde -2% PFA	50-100 nm	For electron microscopy[19]

Histological Stains

Many histology stains were developed for the accurate imaging of the cellular structure of tissues and organs. Currently, there are many different histology stains in use to detect particular cells and components in different types of biological tissues[1]. Hematoxylin and Eosin (H&E) staining is the most frequently used. Hematoxylin can bind to acidic structures and stain the nuclei blue, while Eosin binds to and stains basic structures pink[20]. It is usually used as a routine stain in histology lab to show the general structure of the tissue. However, there are many other histology stains commonly used for some specific types of cells or tissues such as Oil Red staining for lipids, Sirius Red staining for collagens, and others. Table 2 lists the most commonly used histological staining methods (Table 2).

Table 2: List of histology stains

Name of Stain	Mechanism and Color(s)	Application	Representative images
Hematoxylin and Eosin (H&E) staining	Hematoxylin can bind to acidic structures and stain the nuclei blue, while Eosin binds to and stains basic structures pink[20].	A commonly used stain in histology labs to show the general structure of the tissue.	
Masson's Trichrome staining	Formed from a mixture of three dyes to stain plasma (red or pink), fibers (blue or green), and nuclei (dark brown to black)[21].	Widely used to differentiate collagen and smooth muscle in tumors and pathologies of some disease[21].	
Mallory Trichrome staining	It uses the three stains: aniline blue, acid fuchsin, and orange G. As a result, this staining technique can reveal collagen (intense blue), cytoplasm and neuroglia (red), and ordinary cytoplasm, nuclei, and red blood cells (yellow)[22].	Used on connective tissue to indicate collagen and reticular fibers.	
Gomori Trichrome staining	Stains are formed from a mixture of three dyes to stain connective tissue and collagen (green or blue), muscle, keratin and cytoplasm (red) and nuclei (grey/blue/black)[23].	Used to identify an increase in collagen fibers in the connective tissue or to differentiate between collagen and smooth muscle fibers.	
Sudan staining (Sudan III, IV, black and oil red O)	Sudan dyes have high affinity to fats. Stain color depends on the dye used[1].	Used for lipids and triglycerides on frozen sections[1].	
Sirius Red staining	Sirius red is an acidic hydrophilic staining that colors collagen fibers in red[24].	The most commonly used stain to evaluate the level of tissue destructuring[24].	
Nissl Staining	Classic nucleic acid staining method used on nervous tissue sections. The active dye in the staining solution can vary, but toluidine blue or cresyl violet are common components[25].	Widely used method to study morphology and pathology of neural tissue.	
Reticulum silver staining	It relies on the argyrophilic properties of the fibers. After adsorbed, it needs a developer to convert the invisible silver salts to a visible metallic silver (black)[26].	A popular staining used to visualize reticular fiber.	

Histological protein immunostaining

There are three main types of histology immunostainings: immunohistochemistry (IHC), immunocytochemistry (ICC), and immunofluorescence (IF). All three techniques utilize antibodies to provide visual details about protein abundance, distribution, and localization[27-29]. However, there are some differences between these techniques. IHC is the most commonly used immunostaining technique, which detects proteins in cells of a tissue section by exploiting the primary antibody's biding specificity to the protein of interest in biological tissues[27,28]. It can reveal the existence and localization of different cells in tissue sections. Thus, it is usually performed on tissue sections, either paraffin-embedded or cryo-sectioned[27,28]. ICC is often used interchangeably with IHC, except in terms of the biological sample that is analyzed. ICC refers to performing the immunostaining on cells grown in a monolayer or in suspension, which are deposited on a slide[30]. Both IHC and ICC use chromogenic reagents to detect target proteins.

On the other hand, IF can be performed on either tissue or cells. Unlike IHC and ICC, IF uses a fluorescence detection method to detect target proteins[28]. As fluorescence comes in many colors, it allows for the detection of several different proteins simultaneously. Hence, IF is ideal for studying co-localization of different proteins[28]. (Table 3) lists the difference among these three immunostaining methods.

Table 3: Comparison of three protein immunostaining methods

	IHC	ICC	IF
Tissue preservation	Perfusion or immersion fixation in formaldehyde or 4% PFA	4% PFA (short time)	Perfusion or immersion fixation in formaldehyde or 4% PFA
Sample type	Tissue sections	Cultured cells isolated cells	Tissue sections
Labeling method	Chromogenic	Chromogenic	Fluorescence
Microscopy	Light microscopy	Light microscopy	Fluorescence microscopy / Confocal microscopy
Advantages	Higher sensitivity, longer lasting signal	Higher sensitivity, longer lasting signal	Suitable for detecting both single protein and multiple proteins, good for protein co-localization
Disadvantages	Only suitable for detecting single protein	Only suitable for single protein	Lower sensitivity, shorter lasting signal

In situ hybridization (ISH) methods

ISH methods are standard histological techniques that allow for localization and detection of nucleic acid sequences within morphologically preserved tissues sections. This allows for the acquisition of temporal and spatial gene expression information and genetic loci[29]. Currently, there are two main methods to detect nucleic acid sequences in ISH: fluorescence (FISH) and chromogenic (CISH)[31,32]. CISH enables the procurement of genetic information in the context of tissue morphology and is usually used in molecular pathology diagnostics[31,32]. While FISH offers the detection of multiple targets simultaneously and visualization of co-localization within the same tissue section[31,32].

Due to the probe-dependence of ISH, selection of the proper probe is critical. Recently developed RNAscope® ISH and BaseScope™ enable detection of a single transcript and endorsement of multiple targets in the same tissue sections[33,34]. The Alkaline phosphatase-based chromogenic and fluorescence detection method for basescope ISH developed by Wang, Y., *et al*. further improved the resolution of the BaseScope™ assay[3]. This method also offers the ability to investigate multiple RNA and protein targets simultaneously on the same tissue sections[3]. Because of its high sensitivity in nucleic acid detection and advantages of detection in morphologic context, ISH has been widely used for clinical cytogenetics, gene mapping, research analysis of nuclear structures, and gene functions. Nevertheless, the number of genes that can be measured simultaneously is still limited.

Advanced next generation histological techniques

Advanced three-dimensional histological techniques

Traditional histological stains are limited to two dimensions. Examining the entire, three-dimensional tissue sample has been a dream of histologists for years. Recently, Busse, M., *et al*. and Muller, M., *et al*., developed eosin-based cytoplasm-specific X-ray staining[5], and hematein-based nucleus-specific X-ray staining[6] methods, which enable high-resolution microCT imaging of whole organs and nanoCT imaging of smaller tissue pieces retrieved from the original sample[5,6]. The eosin staining and hematein staining offer improved contrast of the soft tissue and is compatible with additional histological methods[5,6]. Along with microCT imaging, a high-resolution three-dimensional rendering of the tissue can be produced[5,6]. In the future, this may allow the production of virtual tissue slices, negating the need for arduous preparation techniques.

Almost at the same time, Lai, H.M., *et al*. developed a method to see through human brain tissue at high resolution[7]. Traditionally, whole-brain imaging can be achieved by cutting brain tissue into thin slices and tracing the nerve fibers over many sections, which is very time-consuming and labor-intensive. To address this issue, scientists developed tissue clearing techniques, which turn opaque tissue transparent and enable three-dimensional

imaging of the brain[8-11]. However, such techniques are not suitable for treating human brain tissues. Lai, H.M., *et al*. developed a unique tissue clearing solution called OPTIClear (Optical Properties-adjusting Tissue Clearing agent) to overcome these barriers[7]. OPTIClear is detergent and denaturant-free, leading to minimal structural changes or damage in the tissue, making it ideal for samples that have been formalin-fixed or paraffin-embedded for extended periods[7]. In combination with fluorescent staining, OPTIClear can be used to produce three-dimensional images of specific regions of interest in human brain tissue. This tissue agent has a potential for widespread application in many histological techniques.

Advanced in situ sequencing techniques

Another advanced histological technique recently developed is in situ sequencing[12,15]. Like in situ hybridization, in situ sequencing can also be performed in fixed cells and tissues[12]. However, unlike in situ hybridization which can only measure limited targets, in situ sequencing can provide a genome-wide profile of gene expression[12] (Table 4). Until currently, several in situ sequencing techniques have been developed, including fluorescence in situ sequencing (FISSEQ)[12], spatially resolved transcript amplicon read-out mapping (STARmap)[13], spatial transcriptomics[14], and Slide-seq[15]. In all these techniques, enzymatic reactions are conducted directly within the original tissue or cells, followed with RNA-seq.

Table 4: Comparison of different in situ hybridization techniques and in situ sequencing techniques

	in situ hybridization		in situ sequencing techniques		
	FISH	CISH	Slide-seq	STARmap	FISSEQ
Labeling method	Chromogenic	Fluorescence	Multi-color fluorescence	Multi-color fluorescence	None
Tissue preservation	Frozen or preserved	Frozen or preserved	Preserved	Fresh or preserved	Fresh-frozen tissue
Preparation time	Less than 2 days	Less than 2 days	2–3 weeks	3 to 4 days from raw sample to final data	Within 2 days
Equipment and unique supplies cost	Low	High	High	Low	Low
Key steps	Sample preparation, probe preparation, hybridization, and detection.	Sample preparation, probe preparation, hybridization, and detection.	Cells/tissue fixation, in situ RT, residual RNA degradation, cDNA circularization, incorporation of primary amines in cDNA, cross-linking the primary amines, cDNA amplification, RCA amplicons are cross-linked to create a highly porous and 3-D nucleic acid matrix.	Sample preparation, SNAIL probes hybridize to intracellular mRNAs, enzymatically replicated as cDNA amplicons, In situ sequencing of DNA amplicons in the tissue-hydrogel complex via SEDAL.	Coverslip coated in DNA-barcoded beads preparation, frozen tissue sections transferred onto the beads, mRNA released from cells was captured by beads, tissue dissolution, reverse transcription and amplification, Mapping of the scRNA-seq.
Gene detection	Single target	Multiple targets but number is limited due to fluorescence limitation	Thousands of mRNAs (and other types of RNA) at once in intact cells	Simultaneously detect more than 1000 genes over six imaging cycles	About 400 unique genes per cell
Advantages	View the CISH signal and tissue morphology simultaneously	Visualize multiple targets in the same sample	3-D, scales to large tissues more efficiently, compare multiple RNA localization patterns in a non-destructive manner	3-D, high efficiency, low error rate, fast processing time, high resolution	3-D, high efficiency, high resolution
Disadvantages	Single-molecule, low efficiency, not applicable to 3-D volumes	Requires long RNA species, lower intensity, low efficiency, not applicable to 3-D volumes	Lack of ribosomal RNA depletion, lack of information on biases, spatial overlap of fluorescence signals, hard to extract meaningful conclusions from plethora of data	Spatial overlap of fluorescence signals, hard to extract meaningful conclusions from plethora of data	Difficult to link the transcriptomes back with their original location at resolutions approaching the single-cell level, hard to extract meaningful conclusions from plethora of data

FISSEQ begins with fixing cells on a glass slide or tissue slides, followed with in situ reverse transcription (RT), and manual sequencing under a confocal microscope[12]. In this progression, each of the four bases is encoded by one fluorescent color and different colored bases of multiple sequences are then read out sequentially within the original tissue[12]. However, due to tissue auto-fluorescence, the efficiency and accuracy of FISSEQ is limited.

Recently, Wang *et al*. developed an intact-tissue RNA sequencing technique in 3-D tissue named STARmap, which integrates hydrogel-tissue chemistry, targeted signal amplification, and in situ sequencing[13]. In this approach, the imaging-based RNA expression information can effectively link with complementary cellular-resolution datastreams describing the anatomy, natural activity, and causal importance[13].

Besides, Stahl, P.L., *et al*. developed another strategy to measure the spatial distribution of transcripts, named "spatial transcriptomics"[14]. In this technique, histological sections are annealed directly to arrayed reverse transcription primers with unique positional barcodes, followed by reverse transcription, sequencing, and computational reconstruction. By this method, they can measure multiple genes within tissue sections and enables novel types of bioinformatics analyses[14].

The most recently developed in situ sequencing technique is Slide-seq, which uses genetic sequencing to investigate the precise cellular structure of tissues and draw detailed, 3-D maps of tissues[15]. Slide-seq begin with a coverslip coated in DNA-barcoded beads with a known position. Frozen tissue sections are then transferred onto the bead monolayer, where any mRNA released from cells is captured. Then the tissue is dissolved, leaving the mRNA bound beads on the surface of the slide. Once tissue digestion is complete, mRNA is reverse transcribed and amplified. Mapping of the scRNA-seq was done by NMFreg (non-negative matrix factorization regression), which reconstructs each scRNA-seq bead expression with the cell type markers from the scRNA-seq data[15]. Due to its efficient, accurate, high-throughput, spatial, and high-resolution characteristics, Slide-seq can significantly facilitate identification of the stem-cell inches, genome-wide spatial gene expression, tissue organization, and function.

CONCLUSION

Histology is vital to studying the structure of biological tissues as well as the understanding and detection of diseases. Although more and more histological staining techniques used for detecting proteins, lipids and nucleic acids have been developed, new more efficient, more accurate and higher throughput histological techniques are still needed. The recently developed advanced high-resolution, high-throughput, and 3-D histological techniques will greatly facilitate histological research. However, the lately developed high-throughput techniques also face the challenge of extracting meaningful conclusions from the plethora of data generated by the advanced techniques. In the future, more accurate techniques are needed to gain amiable data.

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CONFLICTS OF INTEREST

There are no conflicts of interest.

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