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3D IMAGING AND QUANTITATIVE ANALYSIS OF INTACT TISSUES AND ORGANS

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Cover image: The image represents a 3D heterogeneous tissue. The left blurry part represents a tissue without tissue-clearing methods and the right part shows a tissue after clearing.

3D IMAGING AND QUANTITATIVE ANALYSIS OF INTACT TISSUES AND ORGANS

THESIS FOR DOCTORAL DEGREE (Ph.D.)

By

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If you just focus on the smallest details, you will never get the big picture right.

-Leroy Hood

To my growing family

ABSTRACT

Embryonic development and tumor growth are highly complex and dynamic processes that exist in both time and space. To fully understand the molecular mechanisms that control these processes, it is crucial to study RNA expression and protein translation with single-cell spatiotemporal resolution. This is feasible by microscopic imaging that enables multidimensional assessments of cells, tissues, and organs. Here, a time-lapse calcium imaging and three-dimensional imaging was used to study physiological development of the brain or pathological development of cancer, respectively.

In Paper I, spatiotemporal calcium imaging revealed a new mechanism of neurogenesis during brain development.

In Paper II, a new clearing method of clinically stored specimens, DIPCO (diagnosing immunolabeled paraffin-embedded cleared organs), was developed that allows better characterization and staging of intact human tumors.

In Paper III, the DIPCO method was applied to determine tumor stage and characterize the microlymphatic system in bladder cancer.

In Paper IV, a novel method for RNA labeling of volumetric specimens, DIIFCO (diagnosing *in situ* and immunofluorescence-labeled cleared onco-sample) was developed to study RNAs expression and localization in intact tumors.

Overall, the aim of the thesis was to demonstrate that multidimensional imaging extends the understanding of both physiological and pathological biological developmental processes.

LIST OF SCIENTIFIC PAPERS

I. Paola Rebellato*, **Dagmara Kaczynska***, Shigeaki Kanatani, Ibrahim Al Rayyes, Songbai Zhang, Carlos Villaescusa, Anna Falk, Ernest Arenas, Ola Hermanson, Lauri Louhivuori, Per Uhlén

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   The T-type Ca2+ Channel Cav3.2 Regulates Differentiation of Neural Progenitor Cells during Cortical Development via Caspase-3
   Neuroscience, 2019, 402, 78-89

II. Nobuyuki Tanaka*, Shigeaki Kanatani*, Raju Tomer, Cecilia Sahlgren, Pauliina Kronqvist, **Dagmara Kaczynska**, Lauri Louhivuori, Lorand Kis, Claes Lindh, Przemysław Mitura, Andrzej Stepulak, Sara Corvigno, Johan Hartman, Patrick Micke, Artur Mezheyeuski, Carina Strell, Joseph W. Carlson, Carlos Fernández Moro, Hanna Dahlstrand, Arne Östman, Kazuhiro Matsumoto, Peter Wiklund, Mototsugu Oya, Ayako Miyakawa, Karl Deisseroth and Per Uhlén

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   Whole-tissue biopsy phenotyping of three-dimensional tumours reveals patterns of cancer heterogeneity
   Nature Biomedical Engineering, 2017, 1, 796-806

III. Tanaka Nobuyuki, **Dagmara Kaczynska**, Shigeaki Kanatani, Cecilia Sahlgren, Przemysław Mitura, Andrzej Stepulak, Ayako Miyakawa, Peter Wiklund, Per Uhlén

   Mapping of the three-dimensional lymphatic microvasculature in bladder tumours using light-sheet microscopy
   British Journal of Cancer, 2018, 118, 995-999

IV. Nobuyuki Tanaka*, Shigeaki Kanatani*, **Dagmara Kaczynska***, Keishiro Fukumoto, Lauri Louhivuori, Tomohiro Mizutani, Oded Kopper, Pauliina Kronqvist, Stephanie Robertson, Claes Lindh, Lorand Kis, Robin Pronk, Naoya Niwa, Kazuhiro Matsumoto, Mototsugu Oya, Ayako Miyakawa, Anna Falk, Johan Hartman, Cecilia Sahlgren, Hans Clevers, and Per Uhlén

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   DIIFCO: A Single-Cell 3D Imaging Method to Analyze RNA and Protein Expressions in Intact Tumor Biopsies
   Accepted in Nature Biomedical Engineering

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LIST OF ABBREVIATIONS

2D	two-dimensional
3D	three-dimensional
CSC	cancer stem cell
CT	computed tomography
DIIFCO	diagnosing *in situ* and immunofluorescence-labelled cleared onco-samples
DIPCO	diagnosing immunolabeled paraffin-embedded cleared organs
ECM	extracellular matrix
EMT	epithelial-to-mesenchymal transition
ESC	embryonic stem cell
FFPE	formalin-fixed paraffin-embedded
HCR	hybridization chain reaction
HVA	high voltage-activated
ITH	intra-tumor heterogeneity
LSM	light-sheet microscopy
LVA	low voltage-activated
MRI	magnetic resonance imaging
RGC	radial glia cell
SPIM	selective plane illumination microscopy
TME	tumor microenvironment
TNBC	triple-negative breast cancers
VDCC	voltage-dependent calcium channels
VZ	ventricular zone

1 INTRODUCTION

1.1 BIOLOGICAL DEVELOPMENT OF TISSUES AND ORGANS

Each human develops from a single fertilized egg cell, called a zygote. During highly complex and precise processes of cellular proliferation, interactions, differentiation and migration, a zygote becomes a multicellular organism with a variety of cell types. Gene expression patterns, cell signaling and epigenetic modifications lead to these processes (Gurdon, 2013). The process of development from a zygote to a multicellular organism is dynamic in both space and time. Spatiotemporal variations generate the diversity of cell types through the activation of genes within a certain tissue at specific times during development (Lewis, 2008). To comprehend the dynamic architecture of tissue, we need to study not only single cells but also their localization, interactions with other cells and environment.

Although the animal kingdom contains a rich variety of species, the basics of developmental processes are evolutionarily conserved (Shubin, Tabin and Carroll, 2009). After fertilization, the zygote cleaves to generate a blastula and goes through the process of gastrulation to form a three-layer structure. Of the three layers, the ectoderm gives rise mainly to the epidermis and the nervous system, the mesoderm gives rise to muscles, connective tissue, blood, and the kidneys and the endoderm gives rise to the gut tube and its appendages. After fertilization cells have the capacity to differentiate into all (totipotent) or nearly all (pluripotent) cell types of the adult body (Evans and Kaufman, 1981; Martin, 1981). Pluripotent embryonic stem cells (ESCs) have the ability to divide, in a process called self-renewal, and differentiate into other cell types (Thomson, 1998). After gastrulation, cells lose their unlimited potency and become multipotent, exhibiting the ability to generate a diverse, but closely related, set of differentiated cell types. Cell determination options become progressively narrowing until reaching the final stage of terminal differentiation (Brüstle *et al.*, 1999). Although some cells, such as adult stem cells, maintain their multipotent ability, most cells become highly specialized types that cannot change fate thereafter (Jiang *et al.*, 2002).

Stem cell differentiation is a highly regulated process, as most cells lose their 'developmental potential'. In the physiological state, the balance between the generation of new cells and cell death is highly controlled. A damaged cell triggers desirable programmable cell death (apoptosis) (Horvitz, 1999). However, each cell has the potential to undergo a set of genetic alterations resulting in abnormal proliferation and escaping the apoptotic pathway. Cells that stop responding to the signals controlling undesired growth and division continue their upregulated proliferation. This process gives rise to tumor development and, as a consequence, may lead to cancer (Hanahan and Weinberg, 2000). Caspases, enzymes that play a crucial role in apoptosis, are also involved in differentiation and neurogenesis (Abdul-Ghani and Megeney, 2008; Fujita *et al.*, 2008). This means that a balance between self-renewal/differentiation and cell death is crucial for proper tissue development. Thus, the best physiological and functioning conditions are maintained in the dynamic state of equilibrium.

1.1.1 Physiological development of the brain

The brain is believed to be the most complex structure in the human body. It is argued that its main region, the cerebral cortex, is responsible for a source of higher intelligence that distinguishes humans from other species. It is critical to comprehensively understand cellular and molecular mechanisms during brain development as they regulate the expansion of the cortical surface.

The central nervous system is formed from the ectoderm layer during embryonic development. The whole process highly depends on neural stem cells and progenitors that have self-renewal and multipotent capacity. The cerebral cortex develops from cells that are organized into a polarized three-layer structure: basal cortical plate, intermediate zone and apical ventricular zone. The most outer layer, a cortical plate, is where the neurons reside and send out their axons. The middle part, an intermediate zone, is where intermediate progenitor cells reside. On the most apical side, a ventricular zone, is where stem and progenitor cells are located (Malatesta *et al.*, 2003). The processes of neurogenesis switches a transformation into this multilayered tissue and gives rise to a new type of neuronal progenitors called radial glial cells (RGCs) (Rakic, 1971; Malatesta, Hartfuss and Götz, 2000; Hartfuss *et al.*, 2001). As they are more fate-restricted progenitors, most of the neurons originate from RGCs. These cells can either divide through symmetric division, which generates two identical daughter cells, or by asymmetric division, which generates one identical daughter cell and a second different cell (Götz and Huttner, 2005). RGCs go through asymmetric division to generate a neuron (or an intermediate cell that divides into neurons) and RGC or symmetric division to generate two neurons (Noctor *et al.*, 2004). To develop a fully functioning brain, all these processes are highly controlled in space and time by different biological mechanisms, such as calcium (Ca^{2+}) signaling.

Ca^{2+} is a well-studied, versatile intracellular signaling messenger. Its activity regulates many processes ranging from egg fertilization to cell death (Berridge, Bootman and Roderick, 2003). Ca^{2+} signaling plays a special role in neural cells (Koketsu, Cerf and Nishi, 1959; Kuba and Nishi, 1976) as well as neuronal development (Yuste and Katz, 1991; Gu, Olson and Spitzer, 1994). Constant high level of Ca^{2+} was shown to be toxic to cells (Orrenius, Zhivotovsky and Nicotera, 2003). Thus, it is crucial to maintain Ca^{2+} concentrations high in extracellular and low in cytoplasmic Ca^{2+} in homeostasis. To send a signal, intracellular Ca^{2+} levels vary in space and time, which are termed Ca^{2+} waves and oscillations. The frequency of oscillations can be decoded and activate specific cellular pathways (Uhlén and Fritz, 2010). Ca^{2+} oscillations can be spontaneous and were observed during proliferation, migration and differentiation in brain development (Blankenship and Feller, 2010; Rosenberg and Spitzer, 2011; Yamamoto and López-Bendito, 2012). A highly organized machinery of channels, pumps, exchangers, receptors, sensors and buffers regulates Ca^{2+} concentrations inside a cell or/and allows transient Ca^{2+} to enter from the extracellular space. One type of channel is called voltage-dependent calcium channels (VDCCs), which are found in the membrane of excitable cells such as muscle, glia and neurons (Yamakage and Namiki, 2002). They are activated by depolarization of membrane potential, allowing Ca^{2+} influx into a cell.

Depending on the intensity of voltage activation, they are classified into high voltage-activated (HVA) and low voltage-activated (LVA) channels. HVAs are further grouped, depending on seven different main and associated subunits, into L-type (long lasting), P-type (Purkinje)/Q-type, N-type (neural/non-L) and R-type (residual) types. LVA channels are only characterized by one T-type (Tiny/Transient) with three different main subunits (Tsien *et al.*, 1988) and are the first to be expressed in developing neurons (Chemin, Nargeot and Lory, 2002). As mentioned above, early in cortical development, RGCs reside in the proliferative ventricular zone (VZ). It was shown that disturbed Ca^{2+} waves propagating through RGCs decreased VZ proliferation during neurogenesis (Weissman *et al.*, 2004). However, the exact mechanism that regulates Ca^{2+} spontaneous activity during neurogenesis is not fully understood.

1.1.2 Pathological development of cancers

Tumor is a heterogeneous mass containing cancer cells and other cell types, such as endothelial cells, infiltrating immune cells, and stromal cells. Tumors are characterized by a complex network of extracellular matrix (ECM), which all define the spatiotemporal differences in a tumor microenvironment (TME) (Gerlinger *et al.*, 2012; Marusyk, Almendro and Polyak, 2012). As both tumor phenotype and environment are highly heterogeneous, different areas of the same tumor can be composed of various cell subpopulations, vascular networks and accompanying cells. All cells play a role in determining a progress of the tumor; hence treatment success may depend on the selection of tumor regions targeted. Some tumors are able to invade surrounding tissues and/or spread (metastasize) to distant areas, leading to cancer disease. Cancer cell invasion and metastasis are also the result of a complex interaction between tumor cells and their microenvironment (Junttila and de Sauvage, 2013).

Tumor heterogeneity occurs at multiple levels: between patients (intertumor), between primary and metastatic tumors in a single patient, and between the individual cells of a tumor (intratumor). Scientists suggest two possible models for intratumor heterogeneity (ITH). The first model is based on natural selection, as stochastic mutations in each cell cause adaptation and selection for the fittest clones of a tumor (clonal evolution model) (Nowell, 1976). The second model proposes that a subpopulation of cancer cells, named cancer stem cells (CSCs), possess indefinite self-renewal ability to begin and maintain tumor growth (CSC model) (Bonnet and Dick, 1997; Reya *et al.*, 2001; Shackleton *et al.*, 2009). CSCs usually residue in small subpopulations and lead to cancer development, increased intratumor heterogeneity, malignant transformation and metastasis (Nassar and Blanpain, 2016). CSCs are characterized by a number of cell surface markers (Lapidot *et al.*, 1994; Al-Hajj *et al.*, 2003; Singh *et al.*, 2004). In addition to self-renewal and differentiation capacities, CSCs were shown to seed tumors when transplanted into an immunodeficient animal host (O'Brien *et al.*, 2007). As CSCs are linked to metastasis and worse prognosis for a patient, it is hypothesized that they may be located close to vessels to enable easy and fast transport to distinct sites in the body (Calabrese *et al.*, 2007).

Blood vessels play a major role in tumor growth and cancer metastasis (Paduch, 2016). When a tumor spreads, it requires the formation of new blood vessels (a process called angiogenesis) to supply cancer cells with nutrients and oxygen to stimulate their growth. Because the process of tumor angiogenesis is not physiological, the newly formed vessels tend to have weak integrity. This means that blood can leak, and cancer cells can easily enter the bloodstream and travel to distant locations (Nagy *et al.*, 2009). The closer cells are to the vessels, the easier it might be for them to be transported and form tumors in distant locations. Several steps must occur before cells enter the bloodstream. Normally, cells are bound to the extracellular matrix by integrins and other adhesion molecules. Proteins such as E-cadherins and other cell-to-cell junction proteins also bind to each other. If changes or loss of structural proteins occur (for example, increased expression of N-cadherins instead of E-cadherins), cells can gain migratory and invasive mesenchymal properties. This process is called epithelial-to-mesenchymal transition (EMT), and it involves profound morphological and phenotypical changes to a cell (Polyak and Weinberg, 2009).

Over the past several decades, pathologists have staged cancers using conventional approaches based on histopathology. It is crucial to properly stage a cancer, as staging determines treatment strategy and patients' chances for survival. In classic histopathology, analyzed size of the tumor is reduced to enable microscopic examination by cutting the tissue into thin slices. This approach provides single-cell resolution information, enabling pathologists to characterize the size, morphology and expression pattern of individual cells. However, it also causes an information gap between two-dimensional (2D) recorded data and the three-dimensional (3D) state of the original tumor. 3D information of cancer can be achieved by diagnostic imaging, such as computed tomography scans (CT) or magnetic resonance imaging (MRI). These images of a body and organs are used to detect tumors, facilitate biopsies or surgical procedures and evaluate treatment strategies. This approach gives a view on the whole tissue but lacks a cellular and molecular resolution. Hence, there is still no established method in clinics to perform volumetric imaging to examine tumors in 3D with single-cell resolution (Figure 1). As tumors are spatially heterogeneous and some basic features, such as vascular structures, can be missed when analyzing in 2D, there is a need for volumetric imaging of human tumors. Having information on the whole molecular picture might help with more accurate diagnosis, personalized decisions and treatment of cancer patients.

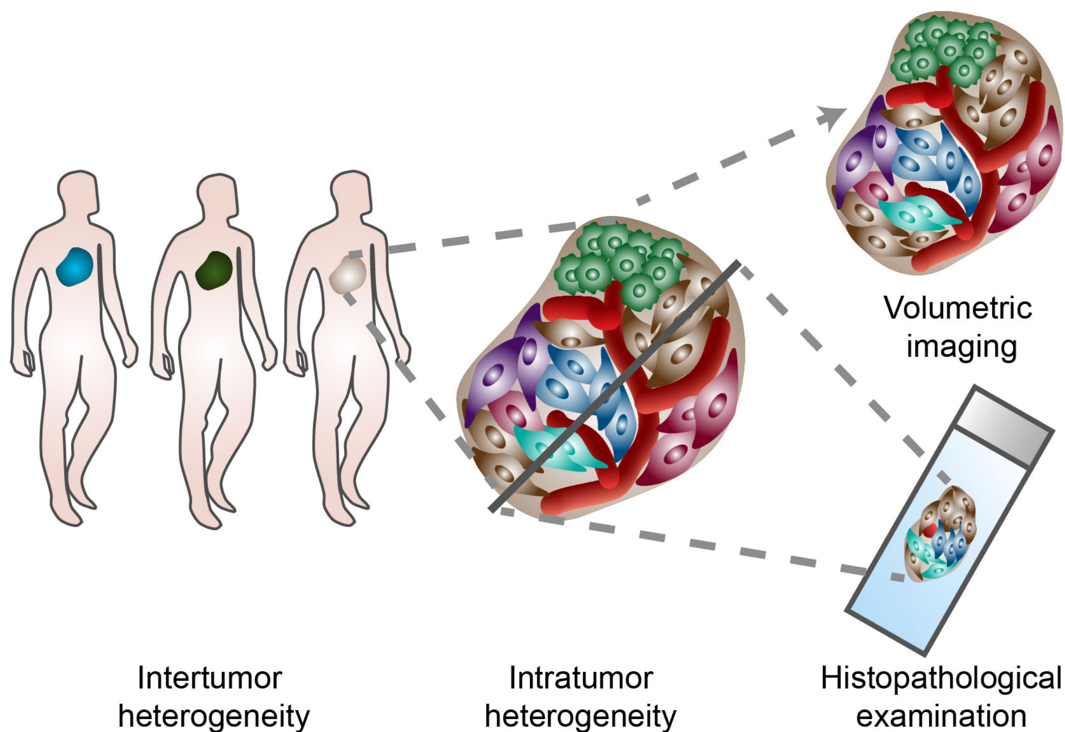

Figure 1. Inter- and intra-tumor heterogeneity could be examined by a classic histopathology and alternative volumetric imaging approaches.

1.2 TOOLS TO EXPLORE BIOLOGICAL DEVELOPMENT

Biological development is a highly dynamic process, yet the precise mechanisms directing it are not fully characterized. One way of studying cellular complexity is through mapping the localization and expression level of RNA and protein at a specific time point. Imaging is the most powerful method to study cellular mechanisms with spatiotemporal resolution. This approach enables both revealing the abundance and localization of proteins/nucleic acids in snapshots or cellular dynamics in real time. Thus, imaging allows multidimensional studies: 2-dimensional (2D) for specific locations, 3-dimensional (3D) for either volumetric or temporal studies or 4-dimensional (4D) when both volumetric and temporal parameters are investigated. To image tissues dimensionally, a few challenges need to be addressed.

Tissues and organs usually have color, making it difficult to look through them. In contrast, each individual cell is translucent and usually too small to investigate with the naked eye. For subsequent cellular dynamics, organisms need to be viable during observation. To image a cell or a tissue, these properties need to be addressed by the use of cell cultures and animals, tissue preparation, labeling and microscopy.

1.2.1 Calcium imaging for dynamics studies

To study tissue development either in 2D or 3D, it is common to fix cells for protein and RNA preservation and for diminishing the fragility of tissues. However, specimen preparation, such as fixing, can introduce distortion to the tissue and reduce dynamics to non-living snapshots of biological processes. Scientists can study fixed samples at different time points to investigate development. However, to understand tissue dynamics, it is crucial to observe how cells respond to biochemical and biophysical changes, which allow them to adapt to a local environment. Hence, we need to study viable cells with spatiotemporal resolution, which pose a challenge for tissue imaging.

One way to monitor the dynamics of cells is to analyze changes in calcium ion (Ca^{2+}) concentrations. As mentioned above, Ca^{2+} is involved in many processes that are essential for the proper development and maintenance of the physiological state of cells. However, calcium also plays a crucial role in apoptosis, which is a process of programmed cell death (Berridge, Bootman and Lipp, 1998). Therefore, understanding the temporal and spatial features of Ca^{2+} signaling in cells and tissues is crucial for gaining knowledge of both the physiological and pathological regulation of systems. Calcium imaging serves as a way to study cellular dynamics, as such imaging is performed at high temporal and spatial resolution. However, cells need to be alive and in physiological conditions to study their Ca^{2+} fluctuations. Given the obvious obstacles in studying calcium activity in living humans, much of the current knowledge is based on cell cultures or *in/ex vivo* animal studies. One approach is to culture cells in a dish, as it is feasible to keep them alive and in nearly physiological conditions. Thus, it is possible to monitor their behavior and processes in a longer time under the microscope. However, cultured cells are in a 2D layer and do not represent the anatomy of mammalian tissues. The other approach is to perform live imaging on embryonic mouse tissues, but it must be kept them in the appropriate conditions throughout the whole procedure. As mentioned above, the exact mechanism of Ca^{2+} signaling in neurogenesis is still unknown. Thus, performing time-lapse calcium imaging in neuronal cells or brain tissues may reveal a mechanism driving the formation of a fully functioning brain.

1.2.2 Tissue-clearing methods for volumetric studies

Although cells are mostly made of water, three-dimensional tissues and organs are intrinsically opaque, which is the greatest challenge in imaging. Thus, for the last several decades, scientists have cut specimens into ultrathin 2D transparent slices (sections) for imaging. This strategy works well if we are only interested in a particular cell or thin portion of tissue as deeper, out-of-focus parts appear blurry without any relevant information. The need to visualize the whole organism has been increasing because cells and organs are three-dimensional structures. One possible solution is to manually analyze slice-by-slice sectioned specimens. Another is to image several thinly cut slices of the specimen to generate a stack of images, which can be then reconstructed using computational tools to generate 3D image. However, cutting whole specimens into thin slices and analyzing every single piece or reconstructing a structure is extremely labor- and time-intensive work. Moreover, classic 2D

approaches may cause the informational gap between slices-based analysis and 3D actual state of a sample. Thus, tissue-clearing methods have been developed, which address lack of tissue transparency and enable 3D examination of tissues.

Diffraction (scattering) of light causes tissue opaqueness, as elements that build a cell and tissue interact variously with light. A cell and tissue is built of lipids, proteins, water and other molecules that bend the light rays disabling light passing through a specimen in a straight line. This property is defined as a refractive index (RI), which indicates how slow light passes through a specimen compared to a vacuum. To visualize a sample, we need to reduce the scattering of light by either diminishing the thickness of a specimen or making it transparent by tissue-clearing methods. Tissue clearing is achieved by matching RIs and allowing the light to propagate in an unobstructed manner, thus making the tissue transparent (Figure 2). It is important to homogenize RIs not only of a tissue but also of a microscope objective and environment, as a mismatch may cause light scattering during imaging. To further improve transparency of a tissue, decolorization (removing pigments), which limits light absorption can be performed. Some tissues contain light absorbing pigments, such as melanin, myoglobin or hemoglobin, which give them a color. The specimens can be decolored by perfusion or by treatment with hydrogen peroxide solution (Tainaka *et al.*, 2014). The use of transparent mutant animals (White *et al.*, 2008) or using microscopies within the electromagnetic spectrum in which tissues do not absorb light (near-infrared window) are also options (Achilefu *et al.*, 2005).

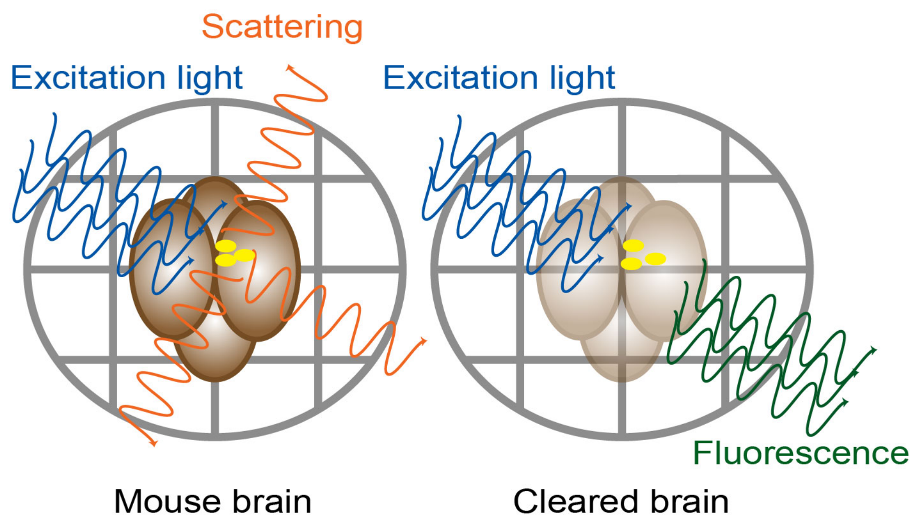

Figure 2. Pathways of light in uncleared and cleared mouse brain.

The first attempt to clear a tissue was already performed more than 100 years ago (Spalteholz, 1914). However, this approach required various dehydration, tissue bleaching and clearing steps with organic solvents, which are considered harsh and damaging for tissues. Since then, many improvements and new methods have been introduced, which use different approaches and key components. The variety of options suggests that there is still no gold standard in tissue-clearing strategy, as they all have advantages and disadvantages.

Tissue-clearing methods face challenges such as lipid and fluorescence tag preservation, immunolabeling compatibility, the need for special equipment, time of clearing, toxicity or altering morphology. Thus, the method of choice depends on the research question and resources.

Group	Methods	Main advantages	Main disadvantages
Organic solvent	**Spalteholz** (Spalteholz, 1914) **BABB** (Dodt *et al.*, 2007) **3Disco** (Ertürk, Becker, *et al.*, 2012; Ertürk, Mauch, *et al.*, 2012) **iDisco** (Renier *et al.*, 2014) **uDisco** (Pan *et al.*, 2016) **vDisco** (Cai *et al.*, 2019)	Immunostaining compatibility No need of special equipment Fast clearing step	Incompatible with fluorescence protein tags No lipid preservation Pretty toxic Morphology alterations (or slight)
High-RI aqueous solutions	**Sucrose** (Tsai, Kaufhold, *et al.*, 2009) **FocusClear** (Chiang *et al.*, 2002) **TDE** (Staudt *et al.*, 2007; Aoyagi *et al.*, 2015; Costantini *et al.*, 2015) **ClearT** (Kuwajima *et al.*, 2013) **SeeDB** (Ke, Fujimoto and Imai, 2013) **FRUIT** (Hou *et al.*, 2015)	Fluorescence protein tag compatibility No morphology alterations Lipid preservation (but Scale) Not toxic	Long clearing step Not as effective for large samples
Hyperhydrating solutions	**Scale** (Hama *et al.*, 2011) **CUBIC** (Susaki *et al.*, 2014; Tainaka *et al.*, 2014)	Immunostaining (CUBIC) and fluorescence protein tag compatibility Not toxic	Long clearing step Morphology alterations
Hydrogel embedding	**CLARITY** (Chung *et al.*, 2013) **PACT/PARS** (Yang *et al.*, 2014)	Immunostaining and fluorescence protein tag compatibility Not toxic Clearing performance	Long clearing step Slight morphology alterations Need of equipment No lipid preservation

Table 1 Groups of tissue-clearing methods and main advantages and disadvantages. Adapted and simplified from (Richardson and Lichtman, 2015).

The clearing techniques are usually grouped into four categories and the first one is based on Spalteholz's organic solvent clearing and the others are based on aqueous solutions (Table 1) (Silvestri *et al.*, 2016). However, all techniques share 2-4 steps, which are: sample pretreatment, permeabilization and/or delipidation, immunolabeling and the final RI matching (clearing) step (Richardson and Lichtman, 2015). The organic solvent group involves dehydration and lipid solvation to clear a tissue by matching to the high RI of proteins: Spalteholz (Spalteholz, 1914), BABB (Dodt *et al.*, 2007), and 3/i/u/vDisco (Ertürk,

Becker, *et al.*, 2012; Ertürk, Mauch, *et al.*, 2012; Renier *et al.*, 2014; Pan *et al.*, 2016; Cai *et al.*, 2019). Most solvent-based high refractive index matching techniques are compatible with immunostaining but not with fluorescence protein tags, as they are quenched during the procedure. These techniques also do not preserve lipids. They do not require special equipment but are pretty toxic to work, with and not all polystyrene and plastic labware can be used. They all influence morphology, but the newest development causes only little shrinkage. Techniques based on aqueous solutions are further divided into high-RI aqueous solutions, hyperhydrating solutions and hydrogel embedding group. High-RI aqueous solution, is based on simple immersion in RI matching solutions: Sucrose (Tsai, Kaufhold, *et al.*, 2009), FocusClear (Chiang *et al.*, 2002), TDE (Staudt *et al.*, 2007; Aoyagi *et al.*, 2015; Costantini *et al.*, 2015), ClearT (Kuwajima *et al.*, 2013), SeeDB (Ke, Fujimoto and Imai, 2013) and FRUIT (Hou *et al.*, 2015). The next group uses hyperhydrating solutions and a lipid removal strategy: Scale (Hama *et al.*, 2011) and CUBIC (Susaki *et al.*, 2014; Tainaka *et al.*, 2014). The last group is based on stabilization by embedding in hydrogel: CLARITY (Chung *et al.*, 2013) and PACT/PARS (Yang *et al.*, 2014). Most aqueous solution and protein-hyperhydration techniques are not toxic and preserve fluorescent protein signals. Some of them are also compatible with immunostaining and can preserve lipids. They can prevent morphological alteration to expansion, but can also make tissue fragile. The biggest drawback is the time of clearing, which can take weeks. Hydrogel embedding techniques have the biggest advantage of preserving both immunostaining and fluorescent protein signals, but they do not preserve lipids. However, such techniques require special equipment to work with, which leads to the need for optimization for each purpose. Moreover, they cause expansion of tissues and the clearing time takes from days to weeks.

Although most tissue-clearing methods were primarily adopted to study the mouse central nervous system, as they enable visualization of complex connections in 3D space and lead to a better understanding of the developing and adult brain (Dodt *et al.*, 2007; Chung *et al.*, 2013; Susaki *et al.*, 2014), the number of tissue-clearing applications is vast. Recently, tissue-clearing methods have been used for profiling tumor biopsies (Kubota *et al.*, 2017; Lee, Bindokas and Kron, 2017; Nojima *et al.*, 2017). Using 3D imaging in clinics can shed a new light on tumor heterogeneity and the microenvironment. However, all tissue-clearing methods mentioned are optimized for basic science applications and tissue storage. Hospitals worldwide have a very established and uniform way of obtaining samples and keeping them for long-term storage; generally, tissues are either kept as fresh samples or formalin-fixed paraffin-embedded (FFPE). As most of the tissue-clearing methods are established on only fresh or fixed tissues, there is a need for a new method that would enable deparaffinization and clearing. Moreover, in basic science, many researchers decrease the size of the tissue to a few millimeters, to decrease the time of antibody penetration, processing and analysis. However, in the clinic, it would be beneficial to visualize and analyze the whole biopsy for better understanding of tumors. Thus, there is a need to establish a 3D imaging technique for intact human tumor biopsies.

1.3 STAINING AND LABELING

Traditional phase-contrast light microscopy enables visualization of cells. However, this approach gives very limited information, such as only the size or shape of a cell. To visualize different types of molecules in the tissue, we need to label the cells using different approaches. The most traditional method is to use dyes, such as hematoxylin and eosin, which have unique affinity for specific subcellular components. Otherwise, protein localization and gene expression patterns can be visualized by immunostaining or *in situ* hybridization, respectively. Moreover, some indicators can measure intracellular ion concentrations in dynamic living cells.

1.3.1 Immunostaining

Antibodies (immunoglobulin) play an important role in physiological immune responses. They are able to recognize and bind antigens, which are foreign substances invading human organisms. This property is used in life science and clinical studies, as follows. Human antigens are injected into host animals to trigger the production of antibodies against this specific antigen. Then, the antibodies are extracted and used to study the location of the antigen within a cell in a tissue. This method is called immunohistochemistry, which is the most common immunostaining technique (Coons, Creech and Jones, 1941). To identify the location of a bound antibody and thus the antigen, antibodies are conjugated directly or indirectly to an indicator such as an enzyme, biotin or a fluorescent molecule. All these strategies visualize the presence of a protein under a microscope, specifying which cells translate the antigen of interest. Thus, immunohistochemistry serves as the most ubiquitous method for studying protein localization.

1.3.2 *In situ* hybridization

To observe the localization of a specific DNA or RNA molecule, *in situ* hybridization can be used which is crucial to fully understand the organization, regulation and function of genes (Gall and Pardue, 1969). Traditionally, *in situ* hybridization of RNA sequences requires that tissues are fixed and cut into very thin slices, and then complementary single-stranded probes (typically synthesized nucleic acids) that target specific RNA are applied. This approach is based on the basic principle of nucleic acid hybridization. A probe is conjugated with a label showing the endogenous location of the complementary RNA sequence. The main challenge of nucleic acid visualization is that they are found in trace amount in tissues, thus require detection amplification. A vast number of *in situ* hybridization methods have been developed, in which nucleic acid amplification involves the use of enzyme and delicate experimental conditions. Recently, a new method was developed, the hybridization chain reaction (HCR), which is isothermal, enzyme-free alternative for the classical approaches. In this method, the probes are amplified to generate a detectable signal. The target initiates a hybridization cascade between two hairpin sequences to generate a fluorescent HCR amplification polymer (Dirks and Pierce, 2004; Choi *et al.*, 2010). The newest development of this method uses split form of the HCR initiator into a pair of probes. Unless two probes bind the target RNA, the initiator will not trigger HCR reaction. In the case of both probes binding to the target,

initiator recruits a pair of fluorophore-labeled HCR hairpins and triggers the growth of a DNA polymer. This improvement omits amplifications of the background signal if the probes bind non-specifically within the sample (Choi, Beck and Pierce, 2014; Choi *et al.*, 2018).

In situ hybridization allows visualization and quantification of RNA expression patterns, which is especially crucial for targets that are not translated into a protein such as non-coding RNA. Although established methods allow protein detection in both 2D and 3D, RNA detection methods are mostly carried out in 2D sections. To date, there have been few attempts to combine *in situ* hybridization with volumetric studies, but they face different challenges such as long protocols or the use of tissue sections (Sylwestrak *et al.*, 2016; Park *et al.*, 2019). Therefore, there is a need for rapid RNA labeling methods in volumetric studies regardless of size, age or storage of samples.

1.3.3 Ion indicators

Cellular structures and molecules are involved in dynamic processes such as movement, reorganization, assembly, and rapid changes in cytoplasmic composition. Both immunohistochemistry and *in situ* hybridizations are typically used on fixed tissues, which take a few hours or days. However, dynamic processes need to be observed in defined timeframe to maintain the activity in living organisms or viable tissues.

Ion concentrations influence different cellular processes in cells and are of great interest in dynamics study, such as neuronal activity. Fluorescently labeled chelators trap ions and can be used to visualize changes in ion concentrations, such as calcium. There are a few available indicators that can be used for calcium imaging. Fura-2 is a ratiometric dye that binds intracellular calcium, and the measurement is calibrated, which provides a stable signal (Grynkiewicz, Poenie and Tsien, 1985). A more recently developed tool, Fluo-3/-4, is a single wave calcium probe for which a higher intensity signal indicates more bound calcium (Kao, Harootunian and Tsien, 1989; Gee *et al.*, 2000).

Immunohistochemistry and *in situ* hybridization enable labeling proteins and RNA, respectively, for determination of localization and abundance in space. Calcium fluorescent dyes are used to observe dynamic processes over time. The last step to observe these phenomena is imaging them with microscopy.

1.4 FLUORESCENCE MICROSCOPY

Once a tissue is prepared by either cutting or clearing and then labeling, the tissue needs to be imaged under the microscope to visualize the cellular staining patterns. Selecting a correct imaging device is as crucial as tissue preparation. Different microscopes have different modalities that profoundly influence imaging. Fluorescence microscopy is the most popular choice to obtain a good quality image with high resolution, sensitivity, contrast and ability to perform quantification as well as for time-lapse imaging. The basic principle of fluorescence imaging is that excitation light is focused on a sample area by the objective lens. A sample is illuminated with light of lower wavelength, which is absorbed by the fluorophores, causing

them to emit light of longer wavelength. However, areas above and below the region of focus are also excited and therefore emit light, which is registered as a blurry image, by a camera. Conventional microscopy, called wide-field microscopy, illuminates a specimen relatively uniformly and is suitable for imaging small and/or live samples such as cell cultures. The biggest drawback is that the entire focal volume is illuminated, and it generates images with both in focus and out-of-focus regions. If a research question involves tissues or thicker specimens, more advanced microscopies are used, which reduces the background noise.

1.4.1 Confocal microscopy

Confocal microscopy imaging addresses out-of-focus challenge and generates in-focus, high-resolution images. First, a pinhole element is the main feature that makes a confocal microscope, which is in the same focal plane as the sample, so it is 'confocal' with the specimen (Minsky, 1988). The general principle is that a pinhole will pass a signal from the focal point but will block signal from outside of the focal point. Second, confocal microscopy enables obtaining 3D high-resolution information about the sample, by using optical sectioning. Optical sectioning is a process of multiple focal plane regions scanning to generate a stack of images that can be reconstructed by a computer to produce clear images of thick samples. In modern confocal microscopes, high-power lasers are used to focus light on a small region of a sample. An image is created, by moving a laser that scans point-by-point over a sample and recording intensity at each spot. Then, a computer reconstructs this information to generate high quality images with good resolution and magnification representing the actual state of the sample. However, this approach causes some challenges that are considered the main drawbacks of confocal imaging. First, it is also relatively easy to quench or bleach a whole sample as the illumination passes through the entire depth of a tissue. Second, volumetric tissues are characterized by high heterogeneity that leads to light scattering or absorption during imaging. The deeper into the tissue imaging is attempted, the lower the probability that light will be focused in a plane. Moreover, confocal microscopy scanning would be extremely slow if it were used to visualize a whole cleared organ or tissue, as it excites region of sample point-by point to generate an image. Thus, confocal microscopy is the best option for high-resolution imaging of 3D, fixed, small slices of approximately 20 μm. As mentioned before, volumetric studies are focused on the tissue environment, such as heterogonous cell populations or vasculature. Thus, there was a need for a more appropriate system to image volumetric samples.

1.4.2 Light-sheet microscopy

Recently, light-sheet microscopy (LSM) has been developed for volumetric imaging. This technology is very rapid, captures 3D dynamic processes and is quenches fewer fluorophores, allowing high-quality imaging of large volumes samples. Similar to tissue-clearing methods, the idea of LSM was developed over 100 years ago and improved with the invention of selective plane illumination microscopy (SPIM); since then, it has been developing rapidly (Siedentopf and Zsigmondy, 1902; Huisken *et al.*, 2004). The basic principle of all LSM technologies is to illuminate a sample with a thin (section) sheet of laser light that is

perpendicular to the detection objective. This means that illumination and detection are decoupled, which is a differentiating feature from other microscopes (Figure 3). Only molecules that are emitted in the illuminated plane are detected, and no out of focus structures are illuminated. To obtain z-sectioning of the entire sample, the specimen needs to be moved through the light sheet beam. The smaller the z-stack steps are, the longer the experiment and bigger the data but also the higher the resolution of imaging. By illuminating a sheet instead of a point, fast volumetric imaging can be achieved for the entire tissue. However, imaging of even cleared tissue generates some light scattering and dense structures hinder the passage of light from the structures behind them, causing shadows and improper illumination of hindered structures. This was initially a challenge in modern LSM due to coherent illumination, as such methods used cylindrical lenses to create a static light sheet (Voie, Burns and Spelman, 1993; Huisken *et al.*, 2004). One way to minimize shadowing and light scattering is to introduce a second illumination arm to improve illumination and image quality (Dodt *et al.*, 2007). Another way is to use a pivot scanner to rotate the light sheet up and down, allowing the illumination to penetrate behind optically dense structures (Huisken and Stainier, 2007). In early studies, the primary application of LSM was in the realm of developmental biology (Huisken *et al.*, 2004; Keller *et al.*, 2008). Currently, a variety of light-sheet microscopy methods are being built around a sample and research question to provide the best image quality, efficiency and resolution for each application.

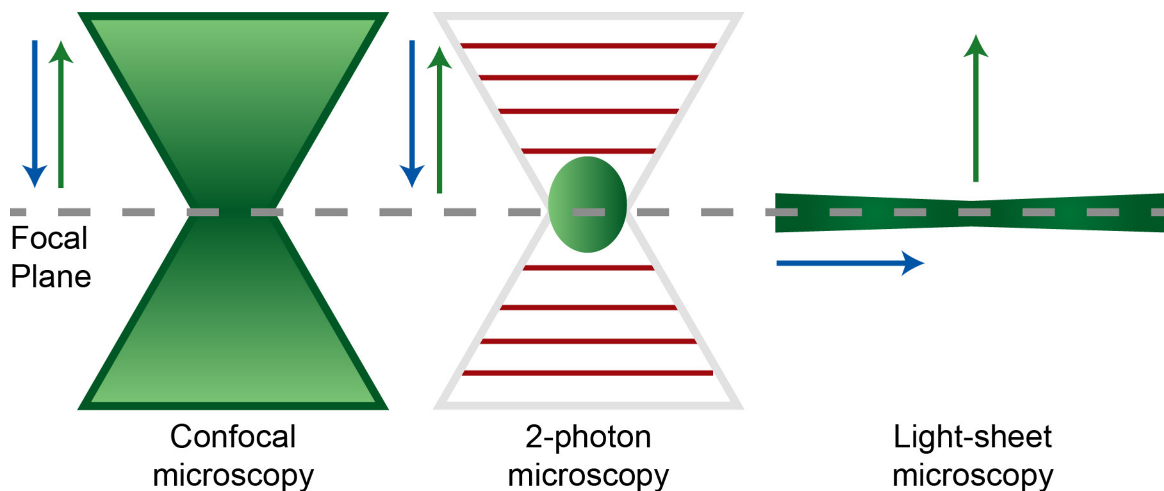

Figure 3 Fluorescent light illuminations in confocal, 2-photon and light sheet microscopy. Blue and green arrows indicate the direction of an excitation or an emission light, respectively. In confocal microscopy, illumination passes through the entire depth of a tissue but is mostly concentrated at the focal plane. In two-photon microscopy, fluorescence is focused in a very narrow spot. In light-sheet microscopy, a thin sheet of a tissue is illuminated and illumination and detection are decoupled.

Although LSM has many advantages, there are some challenges that need to be addressed. First, the refractive indices truly need to match between the sample and the surrounding imaging environment to obtain high-quality images. Second, some custom-built microscopes can be difficult to assemble and maintain, ultimately requiring engineering support. However, commercial LSMs are not always available in core facilities and may be expensive to buy. Finally, most LSMs vary in magnification from 2x to 20x, so the resolution is compromised. Taken together, LSM is most suitable for large volume imaging with slightly lower

resolution. LSM would be the fastest and the easiest to acquire data from samples such as living zebrafish, whole mouse brains or tumor biopsies. For visualization of dynamic processes over time within a whole organism, transparent embryos or transgenic models can be used (Keller *et al.*, 2008; Swoger *et al.*, 2011). However, if we want to image a piece or all of a mouse brain, we need to perform tissue clearing to enable visualization deep inside the tissue. As mentioned before, tissue-clearing protocols involve harsh treatment that is not compatible with living tissue.

1.4.3 Two-photon microscopy

Two-photon microscopy can be used to perform live imaging of thicker samples such as brain slices. Two-photon microscopy is based on the idea that fluorescent molecules are excited by two photons of low energy, such as far-red or near-infrared light, instead of one photon of high energy. If two photons arrive nearly simultaneously, then the energy will add up and excites fluorescence in a tissue (Denk, Strickler and Webb, 1990). The longer wavelengths not only reduce background noise, as light is not absorbed by biological molecules, but can also penetrate deeper in a tissue. This type of microscopy requires extremely high excitation power, as photons of low energy are involves, so a pulsed laser at high peaks is used to avoid photobleaching. Thanks to this approach, fluorescence is excited at a very tight focus. As multiphoton microscopy does not excite out-of-focus, there is no need for pinholes to reduce background noise. Overall, two-photon is suitable to observe dynamic processes of tissues such as thick slices of adult mouse brain, which need to be viable for imaging.

With either time-laps or/and volumetric imaging, we generate multidimensional data that lead to a challenge of analysis and storage. The data enlarge exponentially with increasing sample size, resolution and recorded time. Analysis of multidimensional data is very demanding because it involves supercomputers for image processing and quantification. The need for automatic image analysis is growing alongside the development of imaging strategies, as manual work may take months or even years of constant investigation.

1.5 IMAGE PROCESSING AND ANALYSIS

The field of ‘bioimage analysis’ or ‘bioimage informatics’ is emerging as a response to multidimensional data acquired by advanced microscopy (Peng, 2008). In principle, bioimage analysis enables automatic, reproducible quantification and statistical analysis of large datasets. Recently, an increasing number of image processing tools and software programs have emerged, such as ImageJ or CellProflier, that enable extraction of quantitative information that answers many research questions (Carpenter *et al.*, 2006; Schneider, Rasband and Eliceiri, 2012). However, they still require manual analysis work that can be very laborious for 3D image analysis. Moreover, if a research question is highly specific, it might be necessary to develop specific strategies and algorithms that use programming language such as R, MATLAB, Python or Java. As writing a code from a scratch may be very challenging task for biologists and as the field of multidimensional imaging is continually developing, there is a constant need to provide and share algorithms. The vast

diversity of biological questions disabled the development of the 'one size fits all' analysis pipeline. However, there is an established image analysis workflow, where computational calculations are performed on an image.

The basic image analysis pipeline is based on image preprocessing (filtering), segmentation and quantification (Ljosa and Carpenter, 2009; Roeder *et al.*, 2012). As images are 2D/3D matrices of values at certain coordinates, image transformations change an image by applying a rule or formula to each pixel or set of pixels. The aim of preprocessing is to reduce noise and blur and facilitate segmentation of an image. Image noise is random, unavoidable perturbations of brightness or color information in images, generating 'unwanted signal', which can be diminished by denoising filters. Blur, which is a systemic error of a loss of contrast in smaller features, can be corrected by a deconvolution process that leads to image contrast restoration (Weigert *et al.*, 2018). For example, an unsharp mask or Gaussian filter can be used to reduce blur or noise, respectively. Image preprocessing facilitates the segmentation process, which is a detection of features, such as cells, in an image. Segmentation can be performed by intensity-based thresholding so that every pixel above certain intensity is assigned to a desired object. This generates a binary image that enables separating the thresholded objects. Segmented features enable quantification of different parameters, such as the size, shape or intensity of each object (Politi *et al.*, 2018).

Multidimensional images require also other types of image processing, such as image registration, stitching, and rendering, as time-laps and z-stacks are recorded. Image registration, which is a process of aligning of images, is sometimes required for comparing two images of the same sample taken at different time points. Stitching is required to combine and generate a dimensional representation of a sample, in case where tiles, individual 3D subvolumes, are produced. Image rendering enables to convey the true 3D nature of the data volume or surface.

Regardless of image analysis pipeline, it is always crucial to perform quality control, as some imperfections introduced into an image, such as breaks or low expression levels, could introduce errors in image processing. Thus, the last and most important parts are to explore and verify the quality of the results to ensure that the calculations are actually consistent with a real state.

As mentioned above, there has been a growing need to perform volumetric imaging and analysis of human tumors. This means that there is also a demand for new pipelines and strategies to analyze the tumor microenvironment and heterogeneity. Features in tumors, such as vessel thickness, length, and radius, can be observed and characterized in 3D. In tumor investigation, it might also be of great interest to determine the number of cells or specific cell populations, as it may suggest the state of tumor development. Calculation of the distance from vessels to cells may reveal the metastatic potential of a cancer. As cancer stem cells tend to reside in small populations scattered in a tumor, determination of niche localization and extent can pave the way for understanding tumor progression. Generating new strategies to

analyze tumor vasculature or heterogeneity in 3D can shed new light on tumor development and oncogenic potential.

2 RESULTS AND DISCUSSION

2.1 DYNAMICS STUDY ON BRAIN DEVELOPMENT

2.1.1 Paper I: The T-type Ca2+ Channel Cav3.2 Regulates Differentiation of Neural Progenitor Cells during Cortical Development via Caspase-3

Paper I concerns the involvement of calcium signaling in neurogenesis during brain development. Spontaneous fluctuations of Ca^{2+} have been linked to cell proliferation, cell differentiation and neurotransmitter specifications (Ciccolini *et al.*, 2003; Malmersjö *et al.*, 2013; Uhlén *et al.*, 2015). However, the mechanism behind spontaneous Ca^{2+} activity in corticogenesis is still unclear. Here, we showed that Ca^{2+} activity mediated by the voltage dependent T-type Ca^{2+} channel $Ca_v3.2$ (α1h), which is encoded by the Cacna1h gene, was crucial for proper brain development. Overexpression of this channel caused increased neurogenesis *in vitro*. By contrast, cortical slices from *Cacna1h*$^{-/-}$ knockout mouse embryos showed decreased spontaneous Ca^{2+} activity and decreased thickness of the ventricular zone. As cellular differentiation shares some physiological processes with apoptosis, we investigated Caspase-3 involvement in neurogenesis (Lanneau *et al.*, 2007). Recent observations revealed that Caspase-3 plays a role in neural development in the proliferative zones that is unrelated to the induction of cell death (Yan *et al.*, 2001). Interestingly, we found that the $Ca_v3.2$ channel regulates Caspase-3 activity and mediates neuronal differentiation, as inhibition of $Ca_v3.2$ by drugs or viral knockdown resulted in decreased Caspase-3 activity that was followed by suppressed neurogenesis. In summary, we demonstrated a novel relationship between $Ca_v3.2$ and Caspase-3 signaling that affects neurogenesis in the developing brain.

2.2 VOLUMETRIC STUDIES ON CANCER DEVELOPMENT

2.2.1 Paper II: Whole-tissue biopsy phenotyping of three-dimensional tumors reveals patterns of cancer heterogeneity

Solid cancers have high intratumor heterogeneity (ITH) and serve as a critical factor for the diagnosis and treatment of patients (Gerlinger *et al.*, 2012). Heterogeneous populations of cells, variation in cell sizes, differences in genetic compositions, and distinctions in phenotypic characteristics are the hallmarks of ITH (Gerlinger *et al.*, 2012; Marusyk, Almendro and Polyak, 2012). Examination of 2D slices may provide incomplete information about phenotypic heterogeneity, tumor microenvironment, vasculature and molecular mechanism underlying cancer progression. The highly heterogeneous environment of tumors and the abovementioned limitation of standard pathology reveal the need to improve molecular phenotyping and histopathological diagnosis of cancer. This is especially crucial when grading cancers as each stage leads to different treatment strategies. A better understanding of human tumor heterogeneity and the environment may not only lead to a more accurate diagnosis but also help to develop more specific drugs. Thus, in Paper II, we developed a 3D visualization method for better characterization and staging of clinically

stored, formalin-fixed paraffin-embedded (FFPE) human biopsy samples, and we termed the method diagnosing immunolabeled paraffin-embedded cleared organs (DIPCO).

First, we revealed tumor heterogeneity by studying EMT and angiogenesis in intact mouse samples using the DIPCO method and light-sheet microscopy (Tomer *et al.*, 2014). Second, the DIPCO method enabled phenotyping of human FFPE tumors to study ITH features and vasculature in 3D. Although genome sequencing enabled exploration of the extent of ITH in various types of cancer (Gerlinger *et al.*, 2012; Wang *et al.*, 2014; Andor *et al.*, 2016), our method provided spatial localization of individual cells. Thanks to this development, we were able to show that adjacent cancer cells could have discrete expression levels of EMT markers, such as E-cadherin, N-cadherin and vimentin. Furthermore, we showed tumor hallmarks such as hypo- and hypervascular niches of microvessels. We also developed an analysis pipeline for counting single cells in an entire tumor. After DIPCO examination, it was possible to re-embed samples in paraffin for long-term storage and further studies. Most importantly, we showed that 3D vessel analysis provides more accurate tumor staging than conventional histological 2D methods. We also assessed whether DIPCO could predict chemotherapy responsiveness, such as treatment with anticancer drugs that contain the metal platinum. By 3D vessels characterization, we revealed clear differences between platinum-sensitive and platinum-resistant tumors. To conclude, our method enabled more accurate diagnosis of the cancer stage and prognosis than what was achieved with previous methods, thus allowing for more accurate and efficient treatment strategies for patients.

2.2.2 Paper III: Mapping of the three-dimensional lymphatic microvasculature in bladder tumors using light-sheet microscopy

Lymphangiogenesis plays a crucial role in tumor development and metastasis, as lymph vessels are considered one of the major pathways for systemic tumor spread (Stacker *et al.*, 2014). In Paper III, we applied the previously developed DIPCO method developed previously (Tanaka *et al.*, 2017) to determine whether 3D characterization of the lymphatic system could improve the accuracy of bladder cancer staging. We used tumors from patients with bladder cancer to examine the lymphatic endothelial hyaluronan receptor (LYVE-1), which is a lymphatic microvessel marker (Padera *et al.*, 2002; Kato *et al.*, 2005). Our 3D method revealed more heterogeneous spatial deviation of the LYVE-1 marker in advanced human bladder cancers and provided more accurate staging than standard 2D histological diagnostic methods. This approach would provide physicians with higher quality of information to establish more accurate treatment plans for bladder cancer patients.

2.2.3 Paper IV: DIIFCO: A Single-Cell 3D Imaging Method to Analyze RNA and Protein Expressions in Intact Tumor Biopsies

Paper IV is a continuation and further development of the DIPCO method (explained in Paper II): here, it was applied to investigate intratumoral heterogeneity in cancers for achieving better diagnosis. In this project, we developed a new method for 3D *in situ* hybridization and immunostaining that we named DIIFCO (diagnosing *in situ* and immunofluorescence-labeled cleared onco-samples) for studying both RNA and protein expression patterns in volumetric

samples (Figure 4). To our knowledge, this is the first reported method that enables the detection of both coding RNAs and noncoding variants in large tissues, such as embryonic mouse brains, whole mouse embryos, adult mouse brains and organoids. DIIFCO also enables *in situ* hybridization multiplexing as well as combining whole mounting immunostaining and *in situ* hybridization in intact tissue samples.

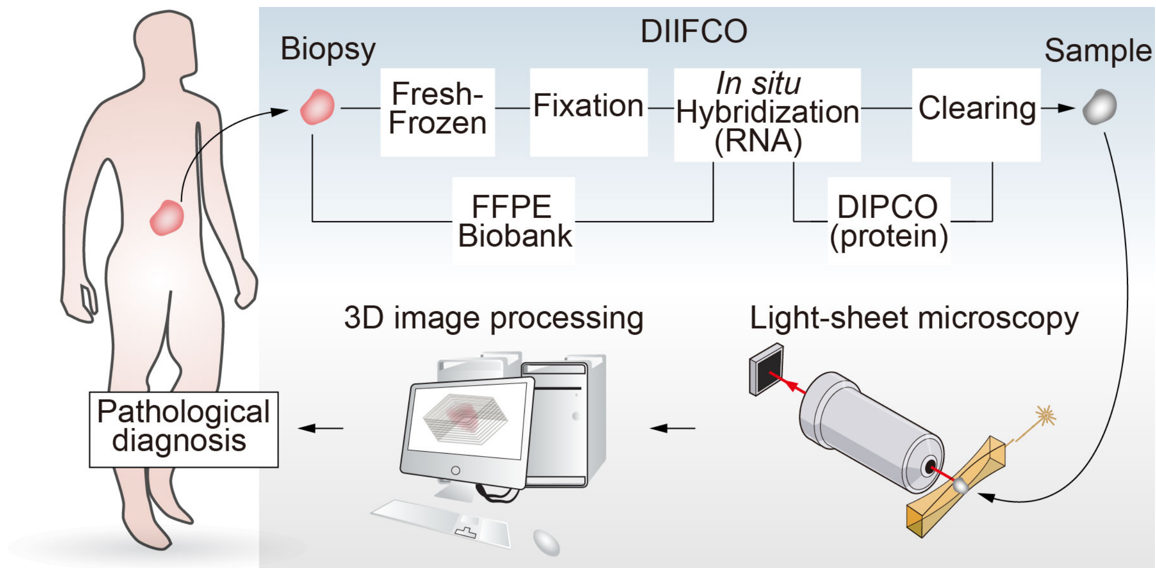

Figure 4. Development of the DIIFCO Imaging Platform (modified from Paper IV).

The DIIFCO method was designed to provide more accurate characterization and diagnosis of FFPE human tumor samples in clinical settings. Thus, we assessed DIIFCO's ability to measure expression levels in single cells to study cancer hallmarks, vasculature and CSC subpopulations in whole tumors. As abnormal expression of some noncoding RNAs plays a role in cancer development (Shahrouki and Larsson, 2012), so we assessed the expression pattern of the noncoding RNA cancer markers UCA1 and MALAT1. We also examined the presence and distribution of CSCs, as they tend to exist in small subpopulations, and can be easily missed in a classic 2D diagnostic approach. To examine CSCs distribution, markers of stemness, such as PROM1, was detected in whole breast tumors and showed heterogeneous expression. We also applied the DIIFCO to explore molecular differences between different types of breast cancers. Some breast cancers do not respond to standard hormonal therapy, as they do not express estrogen and progesterone receptors as well as excess HER2 protein. Those triple-negative breast cancers (TNBC) are considered to be more aggressive and have a poorer prognosis than other types of breast cancer. The DIIFCO method revealed a higher percentage and density of Prom1-positive cells in TNBC samples than in non-TNBC samples. By developing a new analysis pipeline, we could also detect spatial niches of Prom1 cells and show that TNBC samples had significantly higher niche density than non-TNBC samples, which could be associated with malignancy. We also examined whether CSCs reside closer to the vessels, as they are involved in cancer metastasis. We showed that cells with stemness-positive markers, LGR5 and MALAT1, were located significantly closer to the blood vessels in colon cancer sample then marker-negative cells.

This multimodal volumetric imaging with single-cell resolution reveals the pattern of tumors that can only be assessed in 3D, which may lead to a better understanding and treatment strategy for cancer patients.

3 CONCLUSION AND FUTURE PERSPECTIVE

To conclude, this thesis argues that imaging is a powerful technique for investigating development both in physiological and pathological states. Four papers addressed a few imaging challenges, leading to a better understating of brain and cancer development. By using time-lapse recording, a new mechanism of neurogenesis was observed. Better diagnosis of cancer was achieved by a new tissue clearing method as well as through the development of a 3D RNA labeling strategy. Thus, it was shown that the current advancements, such as high-resolution 3D and time-lapse imaging, could reveal highly spatial and dynamic processes during tissue and organ formation. Although the field of multidimensional imaging is growing rapidly, there are certain improvements that still need to be made, such as more robust labeling, imaging and data analysis strategies.

In physiological states, such as brain development, a highly complex process with ever-changing structures and actions cannot be fully understood from static images. In this thesis, time-lapse recording data shed light on brain development. As dynamic processes such as cell development are crucial for fully understanding biological systems, there is a growing need for techniques enabling visualization of entire organisms in real time. Recently, great improvement in depth penetration enabled imaging of developing *Drosophila*, zebrafish and even mouse embryos (Royer *et al.*, 2016; McDole *et al.*, 2018). On the other hand, the use of nanobodies and the development of solvent-based tissue clearing enabled visualization of a whole mouse body with high resolution (Cai *et al.*, 2019). LSM technology is constantly improving, for example, with the mesoSPIM initiative enabling open-source LSM for isotropic imaging of cleared large-scale samples (Voigt *et al.*, 2019). As tissue clearing is a harsh procedure, there is still no technique enabling the visualization of adult organs with spatiotemporal resolution.

In pathological states, such as cancer formation, it is also beneficial to use 3D imaging, as shown in the thesis, which can lead to better characterization and consequently to more accurate staging of cancers. Additionally, features that are missed in classic histopathology, such as lymphatic and blood vessel structure, distance of cell to vessels or localization of specific cell niches, can greatly contribute to understanding cancer. Wide implementation of 3D imaging for diagnosis and prognosis is crucial. This involves collaboration between pathologists and researchers to facilitate imaging and analysis of tumors as well as development of simpler microscope light-sheet microscopes for a rapid diagnosis (Glaser *et al.*, 2017). Using powerful machine-learning approaches and automatic, established pipelines for image analysis would greatly contribute to faster and more accurate diagnosis of cancer.

As tissues are highly heterogeneous structures, combining cell type functions with their locations is of great interest. Thus, we also developed 3D multiplexed *in situ* hybridization as well as to combine immunostaining with *in situ* hybridization in various volumetric samples. Studying both protein and RNA expression can shed light on cellular differentiation or carcinogenesis and can be used to create study models or to define biomarkers as well as drug

targets. However, to have a full picture of development, function and diseases, we need to link cell types to morphological, physiological and behavioral features. Thus, combining molecular fingerprints to tissue localization would help to fill this gap. Imaging a tissue under the microscope can identify cell types with spatiotemporal resolution.

Spatial transcriptomics is a rapidly growing field that allows single molecule RNA spatial detection and multiplex analysis of cellular transcripts. Various microscopy-based methods, such as cyclic FISH, barcoded FISH and *in situ* sequencing, have been developed (Raj *et al.*, 2008; Ke *et al.*, 2013; Lubeck *et al.*, 2014; Chen *et al.*, 2015; Shah *et al.*, 2016; Codeluppi *et al.*, 2018). Although the detection efficiency and gene throughput are high with certain FISH methods covering almost the whole transcript, the spatial throughput is usually limited. Spatial limitations are based on optical density, microscopy capacity and generation of large datasets. Some approaches to overcome these challenges have been made, such as combining spatial transcriptomics methods with expansion microscopy (Wang, Moffitt and Zhuang, 2018). This approach addresses the optical density of the probes in the tissue but leads to larger samples and difficulties with imaging and analysis. Performing 3D, multiplex, single molecule RNA detection in intact organs would face all of these problems. However, organ-wide patterns of gene expression with a single-cell spatial resolution would help a better understanding of biological functions, networks, and interactions between different cell types and lead to understanding of morphogenesis during organ development and maintenance. The first report of such a study was published very recently, and it showed that spatiotemporal organ-wide gene expression leads to a better understanding of human heart development (Asp *et al.*, 2019).

On a protein scale, spatial proteomics enables linking protein subcellular localization to protein function in physiological and pathological states. Some tissue-clearing techniques are compatible with dyes and antibody labeling, but some techniques purge the epitopes of antibodies, leading to labeling with only a limited number of applicable antibodies. Thus, the development of new strategies enabling deep-tissue labeling with full epitope preservation in the whole body is of great interest. Such an approach has been made by applying nanobodies for protein labeling (Cai *et al.*, 2019). The vDisco method also scales up immunolabeling to a whole mouse body, paving the way for multiplex protein labeling in body-scale analysis. In the future, performing either multiround nanobody staining or multiround detection of nanobodies could provide a platform for volumetric spatial proteomics.

We are also in need of standardized organ and whole body atlases that enable comparison of RNA and protein expression in different individuals. For example, the CUBIC-X method was used to expand the adult mouse brain, enabling whole-brain profiling with single-cell resolution and the creation of a CUBIC Atlas (Murakami *et al.*, 2018). This can be applied for mapping cell activity, types and connections in the brain as well as offering a protocol that could be used to create various organ atlases. VesSAP is a deep-learning-based method that enables segmentation and analysis of the brain vasculature of a whole mouse brain. It can serve as a vasculature brain atlas leading to progress in understanding the brain (Todorov *et*

al., 2019). In the future, the generation of atlases for the whole mouse as well as human organs would be of great interest.

If the development of tissue clearing methods, labeling and microscopy continue to progress, we could visualize all human organs in an intact state. The SHANEL method aimed to overcome the challenges of intact adult human samples, and it successfully revealed the structural details of various human organs successfully in an unbiased way (Zhao *et al.*, 2020). This or other prospective method could be used to clear, label, visualize and analyze the whole human body, which would lead to a holistic way of understanding physiology or treating patients.

High-resolution, large-scale and time-lapse recordings generate challenges for data analysis and storage. With the advancement of imaging techniques, the need for new, automatic and powerful computational approaches will grow proportionately. For example, DeepMACT technology was recently established, and it enables detection of cancer metastasis and drug targeting at the cellular level in the whole mouse body. Here, an AI-based method could speed up the discovery of effective cancer treatments (Pan *et al.*, 2019). In the future, further development of machine learning pipelines could pave the way for faster, unbiased and more accurate analysis of multidimensional samples.

In summary, the co-occurrence of tissue clearing, labeling, advanced microscopy and image analysis leads to unbiased, volumetric, spatiotemporal studies, which accelerate the discovery of developmental as well as physiological and pathological mechanisms for whole organisms.

4 POPULAR SCIENCE SUMMARY (IN ENGLISH AND POLISH)

English

This thesis focuses on imaging methods that enable visualization and analysis of tissue and organs in 3D over a period of time, which leads to a better understating of tissue development and maintenance.

Organs and tissues are highly complex structures that come together to form whole organisms. Scientists have spent hundreds of years fully examining elements of the human body, yet little is known about its complexity. The main reason is that organs and tissues are highly heterogeneous. Each organ has a three-dimensional (3D) architecture, which is composed of various cells and environments that may change over time. This is why the features of organs/tissues need to be investigated in space and time to fully comprehend their organization and functions. If we look at an organ, we can see its gross anatomy, but we cannot see its cellular and molecular composition as well as dynamic changes. Although the human body is mostly made of water, we do not see what is deep inside organs as they are not transparent. The reason is that each cell is composed of different molecules, such as proteins, lipids and cytosol. Their unique properties cause bending (diffracting) of light when it is passing through an organ. As our body is made of billions of various cells, the light is highly diffracted, which causes tissues to be opaque. Additionally, most cells are too small to see with the naked eye. Thus, imaging is a great tool to obtain detailed information with spatiotemporal resolution. Although there are many well-established methods of imaging, challenges persist.

The standard procedure for tissue imaging is first to cut a sample into small slices to see structures deep inside it. Then, elements of cells (proteins and RNAs) are labeled with a fluorescent tag and visualized under a microscope. These tags absorb the light and emit a visible signal. However, most microscopes have a limited imaging depth, meaning that only several dozen micrometers of a tissue surface can be visualized. Thus, as mentioned above, the standard procedure involves two-dimensional (2D) slices of tissues. Cutting even a small piece of an organ is very labor- and time-intensive work. Moreover, 2D slices do not represent real volumetric architecture. Understanding an organ/tissue in 3D is crucial, for example, as pathologists who diagnose cancer patients may miss some determining features if they investigate only a few thin slices of a tumor biopsy. Moreover, standard 2D procedures involve slices, which are not viable. Understanding the cellular dynamics that shape organs/tissues requires imaging living cells over a period of time.

The first aim of this thesis was to visualize a whole tissue without actually cutting it into smaller pieces. The second aim was to develop a method to label not only proteins but also RNA in large samples, which has not yet been achievable. After labeling both proteins and RNAs, we made the tissues transparent by ‘clearing’ them with a set of chemicals reduce the diffraction of the light. Next, we chose a microscope that was fast and efficient to scan a few

centimeters of tissue in a few hours. As a last step, we stored a large number acquired of images, visualized the results and performed quantitative analysis. We have developed this method for clinical human tumor biopsies that are stored in a very established and specific way. Human tumors are highly heterogeneous tissues with various populations of cells existing in specific areas. These distinct populations as well as their environmental features, such as blood vessel compositions, can only be properly examined in 3D. Thus, by using algorithms and special software, we discovered new tumor characteristics. In comparison to standard 2D strategies, our new methods improved the characterization and staging of cancer and consequently may lead to more accurate diagnosis and treatment strategies for patients.

The last aim was to examine tissue dynamics over time. Since it is estimated that we are limited to understanding only a small percentage of brain functionality, we used imaging techniques to investigate brain development. As cells needed to be viable for this analysis, we used living tissues dissected from mice and cell cultures in a dish. We have found that certain proteins contribute to spontaneous calcium signaling, which is one of the most important ways for cells to communicate with each other. This process stimulates the creation of new neurons in the developing brain.

To summarize, the imaging in space and time performed in this thesis led to a better understanding of physiological and pathological development.

Polish

Głównym celem tej pracy doktorskiej jest zrozumienie biologicznego rozwoju i funkcjonowania tkanek i narządów człowieka poprzez zastosowanie metod obserwacji mikroskopowych trójwymiarowej i na przestrzeni czasu.

Ciało człowieka zbudowane jest z tkanek i organów o skomplikowanej budowie. Wiele lat badań zostało poświęconym studiom nad niezależnymi elementami ludzkiego ciała, ale niewiele wiadomo na temat organizmu w całej jego złożoności. Tkanki i narządy są trójwymiarowe (3D) i bardzo zróżnicowanie pod względem kompozycji komórkowej i środowiskowej, która dodatkowo ulega zmianom w czasie rozwoju. Dlatego tak ważna jest analiza struktur biologicznych w 3D i na przestrzeni czasu, aby w pełni zrozumieć ich budowę i funkcję.

Kiedy spojrzymy na wybrany organ możemy opisać jego podstawową anatomię, jednakże nie jesteśmy w stanie dostrzec jego składu komórkowego i molekularnego, a także przeanalizować dynamicznych zmian. Ciało człowieka nie jest przezroczyste pomimo, że jest w większości wypełnione wodą. Każda komórka zbudowana jest z różnych cząsteczek, takich jak białka, tłuszcze czy płynne składniki cytoplazmy, których zróżnicowane właściwości powodują zaginanie (dyfrakcję) światła przechodzącego przez narząd. W związku z tym, że ciało budują miliardy komórek o rożnym składzie i właściwościach, światło ulega silnej dyfrakcji. To z kolei powoduje, że tkanki są nieprzezroczyste. Ponadto, większość komórek jest zwyczajnie zbyt mała, aby można je było zobaczyć gołym okiem.

Zatem obrazowanie z użyciem mikroskopu jest doskonałym narzędziem do uzyskiwania szczegółowych informacji w czasie i przestrzeni. Pomimo, że technika obrazowania istnieje już od wielu lat, jej praktyczne zastosowanie wciąż stanowi ona wyzwanie dla naukowców.

Standardowa procedura obrazowania tkanek obejmuje pocięcie materiału biologicznego na cienkie, kilku-mikrometrowe skrawki. Następnie elementy komórkowe (białka i kwasy rybonukleinowe) barwi się fluorescencyjnym znacznikiem i obserwuje pod mikroskopem. Te znaczniki absorbują światło i w konsekwencji wysyłają widzialny sygnał. Jednak większość mikroskopów ma ograniczoną głębokość obrazowania. Oznacza to, że można zaobserwować jedynie kilkadziesiąt mikrometrów powierzchni tkanki. Analiza takich dwuwymiarowych tkanek jest praco- i czasochłonna, a dodatkowo nie odzwierciedla prawdziwej trójwymiarowej architektury tkanek. Zrozumienie narządu i tkanki w 3D jest kluczowe, ponieważ lekarze, którzy diagnozują pacjentów z rakiem, mogą przegapić pewne decydujące cechy i postawić nieprawidłowa diagnozę, jeśli zbadają tylko kilka cienkich wycinków biopsji guza. Ponadto, taka standardowa procedura polega na analizie tkanek, które nie są żywotne. W związku z tym nie jest możliwa obserwacja dynamicznych procesów komórkowych, które można zbadać tylko w żywych organizmach.

Pierwszym celem tej pracy doktorskiej było zobrazowanie całej tkanki lub narządu w 3D bez potrzeby ciecia jej na skrawki tkankowe. Drugim celem było opracowanie metody, która umożliwia znakowanie nie tylko białek, ale także kwasów rybonukleinowych (RNA) w całych tkankach i narządach, co nie było do tej pory możliwe. Po znakowaniu zarówno białek, jak i RNA, uczyniliśmy tkanki przezroczystymi, „oczyszczając" je za pomocą zestawu odczynników chemicznych, które zmniejszają dyfrakcję światła. Następnie wybraliśmy mikroskop, który jest szybki i wydajny do obrazowania kilku centymetrów tkanki w ciągu kilku godzin. W ostatnim etapie zapisaliśmy na superkomputerach tą dużą liczbę uzyskanych obrazów, zaprezentowaliśmy wyniki i przeprowadziliśmy analizę ilościową.

Opracowaliśmy tę metodę z myślą o biopsjach guzów ludzkich, które są przechowywane w bardzo proceduralny i specyficzny sposób w szpitalach. Guzy ludzkie są wysoce zróżnicowanymi tkankami z różnymi populacjami komórek istniejącymi w określonych obszarach. Te odrębne populacje, a także ich cechy środowiskowe, takie jak kompozycje naczyń krwionośnych, można właściwie zbadać tylko w 3D. Stosując więc algorytmy i specjalne oprogramowanie komputerowe, odkryliśmy nowe cechy guzów. W porównaniu ze standardowymi strategiami 2D nasze nowe metody poprawiły charakterystykę i ocenę stopnia zaawansowania raka, a tym samym mogą prowadzić do dokładniejszej diagnozy i strategii leczenia pacjentów.

Ostatnim celem tej pracy było zbadanie dynamiki rozwoju tkanek w czasie. Zastosowaliśmy techniki obrazowania w celu zbadania rozwoju mózgu, ponieważ szacuje się, że do tej pory rozumiemy jedynie niewielki odsetek jego budowy i funkcji. W celu przeprowadzenia obrazowania mikroskopowego w określonym czasie, musieliśmy użyć żywotnych tkanek lub komórek. W tym celu wykorzystaliśmy mózgi myszy laboratoryjnych lub kultury

komórkowe. Odkryliśmy, że niektóre białka przyczyniają się do spontanicznej sygnalizacji wapniowej, która jest jednym z najważniejszych sposobów komunikacji między komórkami. Proces ten stymuluje tworzenie nowych komórek, nazywanych neuronami, w rozwijającym się mózgu.

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