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ILLUMINATING TISSUE ORGANIZATION BY IMAGING THE SPATIAL TRANSCRIPTOME

Lars E. Borm

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Illuminating tissue organization by imaging the spatial transcriptome

THESIS FOR DOCTORAL DEGREE (Ph.D.)

By

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Dedicated to granddad Herman, who encouraged me to look inside the black box.

Ultimately, all things are known because you want to believe you know.

Frank Herbert

POPULAR SCIENCE SUMMARY OF THE THESIS

Our bodies are built from cells. These are small functional units that work together to form our tissues, organs and ultimately us. Taking a closer look at these cells reveals that not all cells are identical and there is a huge diversity in types of cells. These types reflect the differences in functionality between cells. For instance, muscle cells are capable of contracting when instructed, cells in our retina can sense light and cells in our nose can detect smells. To enable the function of a cell, the cell needs to have certain functional proteins. Muscles have proteins that can contract, retina cells have proteins that can interact with light and olfactory neurons in our nose have receptors that can bind to molecules floating in the air.

The instructions of how to build a specific protein can be found in the DNA of the cell, where each protein is encoded in a gene. All cells in the body have the same DNA, yet the cells are different. This is because the DNA also encodes a regulatory code that instructs which genes are active in which type of cell. When a gene is activated a copy of the gene is made in a molecule called RNA, which is then used as a template to make the functional protein.

If we want to know what function a cell has, we can look at which proteins or RNA it is producing. For instance, light-sensing proteins or RNA will only be found in retina cells. Thus, if we find these molecules in a cell, it must be a retina cell. However, doing this practically is challenging because there are thousands of cell types in our bodies, and it is difficult to detect the activity of so many distinct genes with conventional methods.

This thesis presents two solutions to this problem. We developed two new technologies that leverage previous knowledge of which genes are expressed in which cells to locate where a specific cell is located in the tissue. By labeling the RNA of those genes with a fluorescent tag we can visualize them using a microscope. Compared to older methods, the advancement here is that we can now visualize the expression patterns of tens to hundreds of genes simultaneously, instead of just three. This complex measurement can then be used to identify the cell type of each cell in the tissue sample.

We applied these methods on the mouse and human brain to study how that large diversity of cell types is organized in these tissues. We observe that many types are only found in certain anatomical locations in the brain. Furthermore, we can use the data to automatically make maps of the brain, that can be used as a reference atlas to study brain function. Lastly, we also study how the brain develops and the high complexity that can be revealed with these technologies is an excellent tool to reveal the intricate patterning and organization that is required to make our bodies.

ABSTRACT

Our bodies consist of a large collection of cells that each have their own function in the organ that they reside in. The cells are grouped by functionality in cell types that arise during development as the result of the gene regulatory network encoded in the genome. With the development of novel single cell technologies, we are starting to understand just how diverse our cells are. In the brain for instance there are at least 3,000 distinguishable types. However, we have little understanding of how all these cell types are spatially organized in the tissue, because conventional labeling and microscopy techniques are incapable of resolving such high complexity in a single experiment.

In this thesis I present the development of two methods that can resolve the cellular complexity and spatial organization of mouse and (developmental) human brain samples. These methods are built upon the concept of cyclic RNA labeling with single molecule Fluorescent *in situ* Hybridization (smFISH) to detect hundreds of gene targets in tissue samples. The resulting RNA localizations can then be used to study spatial gene expression and to identify the cell type of each cell in the sample. The cellular identity and position can then be used to study spatial relationships between cells to understand the tissue architecture.

To place the development of these two methods into context, I will first review the field of spatially resolved transcriptomics. I will discuss the methods that are based on microscopy and spatially tagged RNA sequencing, where I will compare their strengths and weaknesses.

Then I will present the two projects:

Paper I presents the development of a cyclic smFISH protocol called osmFISH that leverages the high detection efficiency of smFISH to measure the gene expression of 33 cell type marker genes in the mouse somatosensory cortex at single cell resolution. We developed the labeling technology, instrumentation and analysis software to enable the study of cellular organization at multiple length scales.

Even though osmFISH and related microscopy-based methods generate high quality data they are limited by the spatial throughput so that only small tissue areas can be processed. In **paper II** I present another method called EEL FISH that uses electrophoresis to transfer the RNA from a 3D tissue section onto a flat surface. The collapsing of one dimension substantially reduces the time needed to image, while retaining the information, so that the complex spatial gene expression profiles of entire mouse brain sections, sub-structures of the human brain and human developmental tissues can be studied.

Lastly, I will discuss these results and look at the future of the field of spatially resolved transcriptomics.

LIST OF SCIENTIFIC PAPERS

I. **Spatial organization of the somatosensory cortex revealed by osmFISH**

   Simone Codeluppi,* ***Lars E. Borm****, *Amit Zeisel, Gioele La Manno, Josina A. van Lunteren, Camilla Svensson & Sten Linnarsson*.

   Nature Methods 2018 November; 932-935, 15(11)

II. **Scalable in situ single-cell profiling by electrophoretic capture of mRNA using EEL FISH**

   Lars E. Borm*, Alejandro Mossi Albiach, Camiel C.A. Mannens, Jokubas Janusauskas, Ceren Özgün, David Fernández-García, Rebecca Hodge, Francisca Castillo, Charlotte R.H. Hedin, Eduardo J. Villablanca, Per Uhlén, Ed S. Lein, Simone Codeluppi & Sten Linnarsson.*

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SCIENTIFIC PAPERS NOT INCLUDED IN THE THESIS

I. Comprehensive cell atlas of the first-trimester developing human brain
Braun, E, Danan-Gotthold, M*;* ***Borm, LE****; Vinsland, E; Lee, KW; Lönnerberg, P; Hu, L; Li, X; He, X; Andrusivová, Ž; Lundeberg, J; Arenas, E; Barker, RA; Sundström, E; Linnarsson, S.*
BioRxiv 2022

II. Spatial tissue profiling by imaging-free molecular tomography
Schede, HH; Schneider, CG; Stergiadou, J; ***Borm, LE****; Ranjak, A; Yamawaki, TM; David, F; Lönnerberg, P; Tosches, MA; Codeluppi, S; La Manno, G.*
Nature Biotechnology 2021

III. Cell segmentation-free inference of cell types from in situ transcriptomics data
Park, J; Choi, W*; Tiesmeyer, S; Long, B;* ***Borm, LE****; Garren, Emma; Nguyen, TN; Tasic, B; Codeluppi, S; Graf, T; Schlesner, M; Stegle, O; Elis, R; Ishaque, N.*
Nature Communications 2019

IV. Cartilage-binding antibodies induce pain through immune complex–mediated activation of neurons
Farinotti, AB; Wigerblad, G*; Nascimento, D*; Bas, DB; Urbina, CM; Nandakumar, SK; Sandor, K; Xu, B; Abdelmoaty, S; Hunt, MA; Ängeby Möller, K; Baharpoor, A; Sinclair, J; Jardemark, J; Lanner, JT; Ia Khmaladze,* ***Borm, LE****; Zhang, L; Wermeling, L; Cragg, MS; Lengqvist, J; Chabot-Doré, AJ; Diatchenko, L; Belfer, I; Collin, M; Kultima, K; Heyman, B; Jimenez-Andrade, JM; Codeluppi, S; Holmdahl, R; Svensson, CI.*
Journal of Experimental Medicine 2019

V. RNA velocity of single cells
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VII. The promise of spatial transcriptomics for neuroscience in the era of molecular cell typing
Lein, E; ***Borm, LE****; Linnarsson, S.*
Science 2017

VIII. Molecular Diversity of Midbrain Development in Mouse, Human, and Stem Cells
La Manno, G; Gyllborg, D*; Codeluppi, S; Nishimura, K; Salto, C; Zeisel, A;* ***Borm, LE****; Stott, SRW; Toledo, EM; Villaescusa, JC; Lönnerberg, P; Ryge, J; Barker, RA; Arenas, E; Linnarsson, S.*
Cell 2016

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LIST OF ABBREVIATIONS

DNA	Deoxyribonucleic acid
RNA	Ribonucleic acid
mRNA	Messenger RNA
RNA-seq	RNA-sequencing
PCR	Polymerase Chain Reaction
GABA	gamma-aminobutyric acid
ISH	*In situ* Hybridization
FISH	Fluorescent *in situ* Hybridization
smFISH	Single molecule Fluorescent *in situ* Hybridization
NA	Numerical aperture
A	Adenine
C	Cytosine
G	Guanine
T	Thymine
RCA	Rolling Circle Amplification
ISS	*In situ* Sequencing
HCR	Hybridization Chain Reaction
3D	Three dimensional
HDST	High-Definition Spatial Transcriptomics

1 INTRODUCTION

1.1 Cellular complexity

The fundamental unit of life is the cell. These are small self-contained units that make up the bodies of organisms, from unicellular species like bacteria or algae to complex multicellular organisms capable of intricate behaviors. Most cells are so small that they cannot be observed by the naked eye, and therefore the development of the microscope played a major role in the discovery of cells. Robert Hooke was the first to describe cells after he saw them through a microscope inspired by a design of Antoni van Leeuwenhoek. Hooke named these units cells after the Latin word *cella* for the small rooms monks lived in. The observation of the compartmentalization of life lead to our current understanding that life is based on a large collection of different cells that each serve a specific role in a multicellular organism. However, with the development of new microscopy technologies, we are only now starting to understand how the great diversity of cells is spatially organized in our bodies.

Nevertheless, apart from the functional differences, most cells share a common building plan of a lipid membrane that envelopes the nucleus, cytoskeleton and organelles of the cell. The nucleus contains a regulatory code and a large collection of genes, called the genome, encoded in DNA molecules. When the regulatory code enables the activation of a gene, it gets transcribed into one or multiple copies of RNA, which are used as template to make corresponding proteins that can enact a specific function. The gene regulatory network determines which genes are activated at which time, and thereby controls which molecules are generated, destroyed or modified depending on the cell’s needs. The activity of the regulatory code is in turn driven by the inputs the cell receives. This input can come from within - where one molecule acts on another molecule inside the cell - or from without, through detection of environmental cues, such as temperature, pressure or ion concentrations. Additionally, these extracellular signals can also come from other cells that can relay information through chemical transduction.

Multicellular organisms use this principle of cellular communication to coordinate collaboration, so that the joint output of the many individual cells defines the activity of the organism. However, the cells of a multicellular organism are not all identical and usually they perform specific tasks within the organism. These specializations of cells follow the principle of division-of-labor and gave rise to the existence of cell types; groups of cells that share a common function defined by the regulatory mechanism encoded in the genome (Achim and Arendt 2014; Arendt 2008; Arendt et al. 2016; Arendt, Hausen, and Purschke 2009).

1.2 Cell types

Similarities and differences between cells have been described since cells were discovered. Most early observations were based on the cell’s morphology and groupings based on this property are generally called morphotypes. A great early example of the study of cell morphology is the work by Ramón y Cajal and Camillo Golgi, who applied Golgi’s staining method that completely fills a few randomly selected brain cells with a silver precipitate. This results in a dark cell that contrasts with the translucent other cells, so that the full morphology of the stained cell can be seen. Cajal and Golgi studied the cells of the brain and found multiple groups of cells that shared morphology but were distinct from other groups, like the hippocampal neurons and the Purkinje cells of the cerebellum (Ramón Y Cajal 1897).

Apart from morphology, cells can also be grouped based on other characteristics such as behavior (contracting muscle cells, phagocytic white blood cells), what they produce (hair cells, hormone producing cells) or what they sense (rod and cone cells in the retina, olfactory cells). The neurons in the brain can also be characterized by their electrophysiological characteristics or the specific neurotransmitter they use for communication. Most cells in our bodies have been described based on one or more of the mentioned properties and attempts have been made to organize these cell types (Vickaryous and Hall 2006). However, without the resolution to study individual cells it is hard to be sure if a group of cells actually all belong to a single type or if contamination of other cells biases the measurement.

Recent advances in single-cell biology enabled the systematic study of the hierarchy of cell types and how the genome encodes this structure (Trapnell 2015). Sequencing the transcriptome of individual cells to capture one output modality of the gene regulatory network, turned out to be a good method to compare individual cells and find similarities between them so that cell types could be identified. This field started with the development of methods that could detect minute quantities of RNA by sequencing (RNA-seq) of individual oocytes(Tang et al. 2009). These methods were later perfected to give a quantitative readout of gene expression per cell, so that expression profiles could be directly compared between cells (Islam et al. 2011, 2014; Kivioja et al. 2011).

Single cell RNA-seq methods are now widely used and all rely on the compartmentalization of individual cells, either using tubes (Hashimshony et al. 2012; Ramsköld et al. 2012), microfluidics (Zeisel et al. 2015), multi-well plates (Picelli et al. 2013; Salmen et al. 2022) or droplets (Klein et al. 2015; Macosko et al. 2015), followed by unique molecular identifier tagging, amplification and sequencing. These improvements in single cell RNA-seq scaled the throughput of the method so that thousands to millions of cells can be processed in reasonable time (Cao et al. 2019; Rosenberg et al. 2018). The maturation of the technology and increased throughput lead to the ambitious project to map all cells in the human body in a project called the Human Cell Atlas (Regev et al. 2018). This ongoing effort would form a baseline of the cell types found in humans, give insight into the structure and hierarchy of cell types and serve as a reference atlas to compare to when studying cells in a diseased state. However, to compartmentalize the cells to tag them for single cell RNA-seq, the tissue first needs to be dissociated, so that all spatial information of where these cells come from is ultimately lost.

1.3 Brain

1.3.1 Major brain cell types

Out of the investigated tissues using single cell methods the brain has proved to be the most complex organ with many different sub-types of neurons. The major groups of cells found in the brain are neurons — subdivided into excitatory and inhibitory neurons — oligodendrocytes, astrocytes, microglia, ependymal and vascular cells.

Neurons perform signal processing and can rapidly communicate with other neurons. The neurons contain three main cellular structures: *Dendrites* which are cell protrusions where neurons receive the bulk of the input from other cells. These signals are then transduced to the *cell body* and axon hillock where the signals are integrated. If the integrated signal is sufficiently strong the cell fires an action potential along the *axon* which can be up to a few meters long to communicate with its connected cells. The action potential is a depolarization of the cell membrane potential by an influx of positively charged ions that is normally kept at a voltage of roughly -70mV inside. This depolarization can travel quickly over the surface of the cell and is responsible for the fast signal transduction of our brain. The axon terminates in one or multiple synapses from which a chemical called a neurotransmitter is released that has an effect on the post-synaptic cell. The effect of this neurotransmitter is dependent on the specific chemical used and the presence of the corresponding receptors on the post-synaptic cell. The specific neurotransmitter and receptors are also major components by which neurons can be functionally classified (Cadwell et al. 2015; Fuzik et al. 2015; Tasic et al. 2016; Zeisel et al. 2015, 2018).

Excitatory neurons use glutamate as a neurotransmitter which opens certain ion channels that let in positive ions such as sodium, potassium and calcium, which cause the post-synaptic cell to depolarize. If the depolarization is above a certain threshold the post-synaptic cell will generate an action potential of its own. Conversely, inhibitory neurons use gamma-aminobutyric acid (GABA) as a neurotransmitter which acts on GABA receptors that let in negatively charged chloride ions and thus hyperpolarizes the cell. The sum of these polarizations and hyperpolarizations determine if the cell will fire an action potential or not. Apart from glutamate and GABA there are a number of other neurotransmitters used by neurons, such as serotonin, dopamine, glycine and noradrenaline.

Oligodendrocytes are cells found throughout the brain but mostly in the white matter. These cells wrap the axons of neurons with a sheet of myelin which is rich in lipids and thereby electrically insulating the wrapped part of the axon. In between myelin sheets there are empty spaces called nodes of Ranvier, where the axon concentrates its ion channels. An action potential that travels along the axon will jump fast from node to node instead of as a continuous depolarization. The oligodendrocytes therefore increase the speed by which signals travel through neurons.

Astrocytes are important cells for the homeostasis of the brain. They regulate blood flow, provide nutrients and recycle neurotransmitters from the synaptic cleft (Nedergaard, Ransom, and Goldman 2003). The brain is separated from the blood stream by the blood-brain-barrier formed by the endothelial cells, pericytes and astrocytes so that the brain is protected from pathogens. This also means that regular immune cells cannot normally enter the brain and therefore the brain has its own resident macrophages called the microglia that are responsible for immune defense.

Lastly, the brain has water-filled ventricles lined by neuroepithelium formed by the ependymal cells. These are ciliated cells that use their cilia to propel the cerebrospinal fluid along the ventricular surfaces. Specialized epithelial cells in the choroid plexus generate cerebrospinal fluid in the ventricles by filtration of arterial blood. The fluid provides cushioning to the brain and is important for draining waste from the brain to the lymphatic system (Iliff et al. 2012).

1.3.2 Cell type complexity

The above-described major cell types can be further subdivided into a number of types with distinct characteristics. The neurons show the highest amount of complexity which is related to their use of the different neurotransmitters and their different electrophysiological characteristics (Saunders et al. 2018; Tasic et al. 2016; Zeisel et al. 2015, 2018). In human, an in-depth study has found over 3300 cell types in the adult brain, where most complexity can be attributed to neurons (Siletti et al. 2022). A group of rare neurons mostly coming from the evolutionary older part of the brain is especially responsible for the high complexity as they expressed multiple neurotransmitters in a combinatorial fashion generating high complexity. Moreover, the authors indicate that this study likely under-sampled these cells because they are so rare. It is therefore likely that the cell type complexity is even larger and further studies are needed to decipher this.

Most of the cell types found in the studies described above on adult brains correspond to terminally differentiated cells. However, there are a few exceptions that add another - more gradual - axis of complexity. For instance, oligodendrocytes form a developmental trajectory from oligodendrocyte precursor cells to myelinating oligodendrocytes for which all stages are present in the brain (Marques et al. 2016; Siletti et al. 2022; Zeisel et al. 2018).

Furthermore, neurons are generally post-mitotic but, in the mouse, there is ongoing neurogenesis in the subventricular zone that generates new neurons which migrate to the olfactory bulb, and neurogenesis in the subgranular zone of the dentate gyrus (Altman and Bayer 1990; Altman and Das 1966; Hochgerner et al. 2018). In humans there is neurogenesis in the dentate gyrus, and the neurogenic capacity of the subventricular zone seems to have its output go to the striatum rather than the olfactory bulb (Bergmann, Spalding, and Frisén 2015; Ernst et al. 2014). However, owing to the great difficulty of studying adult neurogenesis in humans, there have been conflicting findings (e.g. (Sorrells et al. 2018) and critical discussion in (Kempermann et al. 2018), followed by several replies). Single-cell analysis methods may provide a means to more definitive answers to this important question.

Studying the cellular complexity of the human brain is challenging due to limited available samples and its high number of cells. Nevertheless, now that single cell RNA-seq methods scale to millions of cells we start to grasp the diversity of the building blocks of our brain.

1.3.3 Development

Most cell types in the brain have an identity that can be traced back to their developmental origin (Siletti et al. 2022; Zeisel et al. 2018). Development is a complex and tightly regulated process that ensures not only that all cells get specified to their adult type but also that all these cells end up in the correct location in the organism.

The brain develops in the embryo from the neural plate that folds into the neural tube. This tube is patterned in the anterior-posterior axis by gradients of morphogens such as retinoic acid, Wnt and its antagonists, FGF and TGFbeta. Orthogonally, the neural tube gets patterned in the dorsal-ventral axis by sonic hedgehog coming from the notochord located ventrally of the neuronal tube that induces the floor plate of the tube. On the opposite side BMPs coming from the neural crest precursors located dorsally of the neural tube induce the floor plate of the neural tube. The combination of anterior-posterior and dorsal-ventral patterning generates an initial specification of brain development, and this process is conserved between chordates. This is further extended during development through morphogen gradients and cell-to-cell signaling.

The anatomical patterning specifies various types of radial glia, which are the progenitor cells for neurons, astrocytes and oligodendrocytes. The radial glia have their cell bodies located close to the lumen of the neural tube and extend a cell process radially outwards to the outer surface of the tube. This radial process forms a scaffold that the daughter cells use to migrate outwards. This process is best studied in the developing cortex of the brain where the daughter cells of radial glia, and in humans also intermediate progenitor cells, generate successive waves of neurons that migrate outwards passing any previous wave.

This way the cortex becomes a layered structure where the deepest layer is generated first and more superficial layers are developed later (Kriegstein and Alvarez-Buylla 2009).

However, not all cells only migrate radially outward to reach their final destination. Some cells also perform tangential migration where cells move orthogonally to the radial processes. The migration is guided by gradients of morphogens or cell-cell interactions that either attract or repulse the migrating cells into the correct location. Continuing the example of the cortex, the inhibitory interneurons found the in the adult cortex, developmentally originated from a different structure called the ganglionic eminence. After they are born, they start the migration to the cortex where they populate it in a layer dependent manner depending on the subtype of interneuron (Faux et al. 2012). In other parts of the brain our knowledge is more sporadic, likely due do the more complex, but still highly organized, anatomical organization of many small brain nuclei in the basal ganglia and brainstem (Anthony et al. 2004; Coulombe et al. 2021).

The importance of the spatial organization of cells in the brain is also highlighted by the severe consequences and developmental defects that arise when cell migration is hampered. For example, a group of radial migration defects in the cortex result in lissencephaly, meaning that the cortex is not folded but smooth. These various lissencephalies are all related to cell cytoskeleton defects caused by mutations in genes such as LIS1, DCX, TUBA1A or TUBB2B. The effects of lissencephaly can be severe where patients can suffer from psychomotor retardation, respiratory problems and seizures.

Collectively, the fact that patterning and cell migration is tightly regulated, evolutionary conserved and the severe defects that arise when cells do not end up in the correct location, strongly indicate that the location of cells is important for proper brain function.

1.4 Locating cells in space

There are multiple methods to locate cells of a certain cell type in a tissue sample. The most common method is microscopy, where you can use a distinguishing feature of a cell type of interest to identify it. Previously, this was mostly done on morphology with labeling method such as the mentioned Golgi stain, or a Nissl stain that shows the cell body shape of neurons and glia. Nowadays, the most common strategy involves the identification of a molecular marker that is uniquely present in the cells of interest, and the (fluorescent) tagging of that particular molecule. The marker usually is a gene that is specifically expressed in the cell type of interest, in which case the RNA transcript of that gene or the protein product can be detected. In the case of RNA, a fluorescently tagged complementary nucleotide probe can be used to label a specific transcript and for proteins a fluorescently tagged antibody can be used.

However, even though microscopy is extensively used to locate cells of interest in tissue samples, it is severely limited in the number of targets it can simultaneously detect. Consequently, the number of cell types it can simultaneously locate *in situ* is also limited, which limits our understanding of the studied tissue. The reason is that the number of fluorophores that can be distinguished based on their emission wavelength, is usually not more than a maximum of 7 in a high-end microscope system, because they would otherwise spectrally overlap. This *plexity*, meaning the number of separate measurements, stands in stark contrast with the thousands of cell types found in our brains.

Thus, we are now starting to understand just how complex the cells of our bodies are, but conventional methods are incapable of resolving how these cells are organized in the tissue to form the organ. Here, I will review the development of new and highly multiplexed methods to solve this problem. Furthermore, I will present two methods that we developed to locate and study the organization of cell types in tissue samples.

2 LITERATURE REVIEW

2.1 Highly multiplexed measurements

To spatially resolve the large variation of cells found in biological systems, we need detection methods that can match this complexity. The last decade has seen a surge of highly multiplexed methods to resolve molecular markers in tissue samples, so that we now start to understand how the large number of cell types are organized in the tissue anatomy and how they relate to each other spatially. Here, I will review the various strategies to map cell identities in tissues, with a main focus on methods that measure the transcriptome of the cells to determine the cell type or state. Multiplexed methods to measure proteins in tissue samples have been developed in parallel and have been reviewed elsewhere (Hickey et al. 2022).

2.2 Imaging

The first category of methods uses microscopy to detect a high number of RNA species *in situ*. This group can be further subdivided into two main branches that either use Fluorescent *in situ* Hybridization (FISH) or *in situ* sequencing to locate RNA in space.

2.2.1 Multiplexed FISH

In situ Hybridization (ISH) is a group of methods that can detect RNA by hybridizing a complementary single-strand DNA probe to a target transcript. This probe can be tagged in a number of ways that allow for visualization using microscopy (Figure 1). The earlies applications of ISH used a radioactive label to detect RNA in cells (Gall et al. 1969; Harrison et al. 1973; John, Birnstiel, and Jones 1969; Wagner and Morrell 1995). Due to the impracticalities related to radioactive labeling, alternative detection using fluorescence (Langer-Safer, Levine, and Ward 1982) and chromogenic labeling were subsequently developed (Jiang et al. 2019; Tanner et al. 2000; Tautz and Pfeifle 1989).

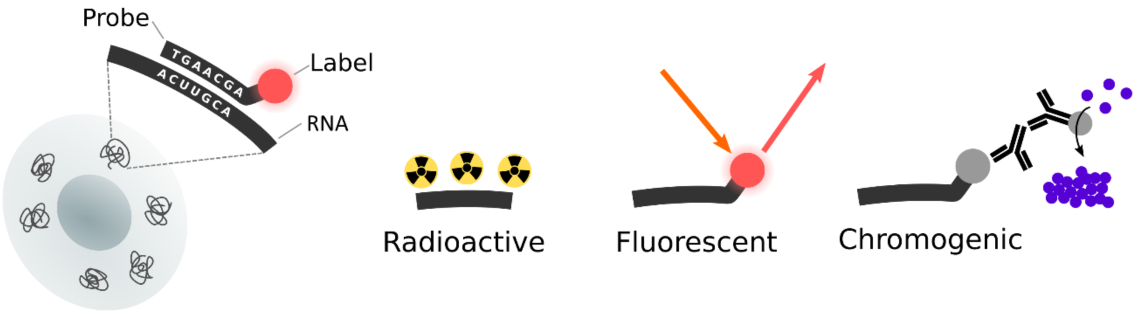

Figure 1| in situ Hybridization with radioactive, fluorescent, or chromogenic labels.

When enough of the labels are accumulated on the target transcript and the background signal is sufficiently low, it is possible to detect individual molecules of RNA with FISH as a diffraction limited spot in the image (Figure 1). This FISH variant is called single molecule

FISH (smFISH) and is highly quantitative because the exact number of transcripts per cell can simply be counted (Figure 2). The first example of this principle used radioactive labeling (Gall et al. 1969) and have since been extended to fluorescent (Femino et al. 1998) and chromogenic detection strategies (Jiang et al. 2019).

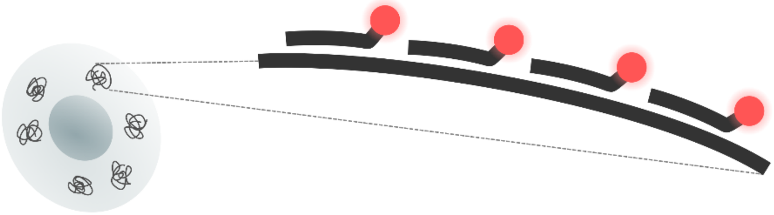

Figure 2| Fluorescent smFISH probes tiling the RNA target.

Especially the fluorescent variant is currently widely used after simplification in probe production, assay speed and the capability to detect multiple targets in the same sample by using fluorophores that emit different wavelengths of light (Itzkovitz et al. 2011; Lyubimova et al. 2013; Raj et al. 2008; Shaffer et al. 2013). Furthermore, commercialization of signal amplified smFISH methods using branched DNA FISH (Kern et al. 1996; Player et al. 2001) or hybridization chain reaction (Choi, Beck, and Pierce 2014; Dirks and Pierce 2004) also contributed to the wide adoption of smFISH.

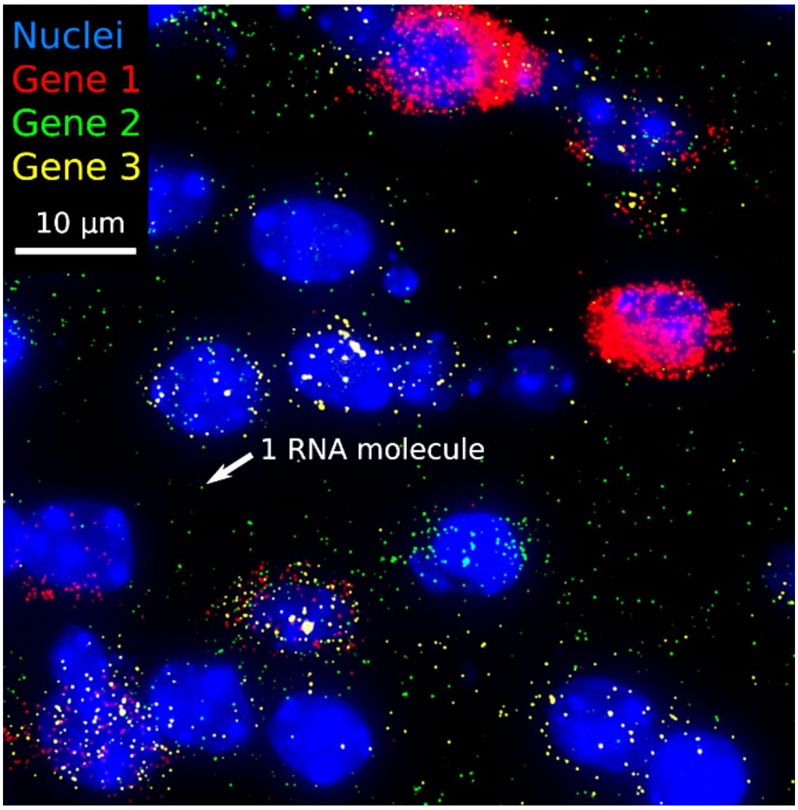

Figure 3| smFISH image for 3 genes with cell nuclei. Each spot is a single molecule of RNA.

To measure the expression of a large number of gene targets with ISH the assay can be parallelized on multiple samples, measuring one gene per sample. This is especially valuable in the case when the samples are highly stereotyped, such as clones of cells in culture or the brains of inbred mouse strains, so that results of parallel experiments can be compared to some extend (Battich, Stoeger, and Pelkmans 2013; Lein et al. 2007). Nevertheless, it would be more valuable if all those measurements could be done in the same sample so that measurements can be directly compared. This would also mean that less samples are needed, reducing the number of experimental animals, or enabling measurements on rare samples such as human tissues, to get the equivalent or even more information.

However, the complexity that can be reached with FISH is limited to typically three to five targets due to the limited number of distinguishable fluorophores. Most fluorophores do not emit a single wavelength of light after excitation but rather a broader spectrum of approximately 100 to 150 nanometer wide. Since typical microscope cameras are only sensitive in the range of 400 to 900 nanometer, the number of distinguishable fluorophores is maximally around seven. However, the fluorophores in the far-red are hard to work with due to dropping camera sensitivity, meaning that the effective number of different fluorophores that are reliably co-detected is commonly four.

Recent advances in labeling strategies are, however, overcoming the *color barrier* to enable measurements of higher multiplexing in the same sample. The firsts solution uses spectral barcoding (Figure 4), where target molecules are labeled with a unique combination or ratio of a limited set of fluorophores, so that the plexity increases to a maximum of 15 targets (Dauwerse et al. 1992; Levesque and Raj 2013; Lubeck and Cai 2012; Nederlof et al. 1990, 1992; Valm, Mark Welch, and Borisy 2012).

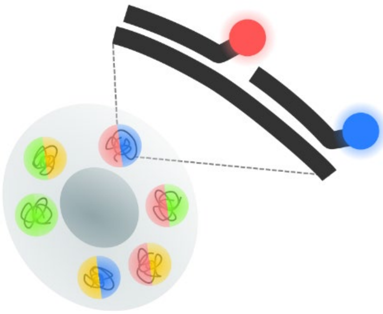

Figure 4 | Spectral barcoding where a single RNA molecule is labeled with one or more fluorphores.

2.2.1.1 *Cyclic labeling*

The real breakthrough in overcoming the color barrier came with the concept of cyclic labeling, where multiple cycles of labeling – imaging – label removal, are sequentially performed on the same tissue sample (Figure 5) (Lubeck et al. 2014). After all rounds are completed, the acquired images can be aligned so that the RNA transcripts can be localized, quantified and studied in the same space.

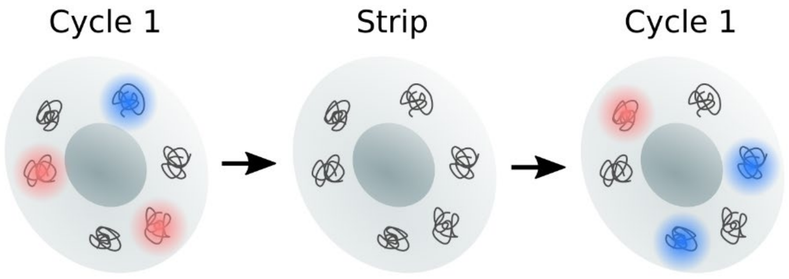

Figure 5 | Linear scaling cyclic smFISH. Each cycle targets a new set of genes with the same fluorophore set. After imaging the fluorescence is removed by stripping to enable another cycle of labeling.

This principle can linearly expand the number of targets, if in each subsequent round the same fluorophores are re-used for new targets, so that the plexity (n) corresponds to the number of fluorophores (f) times the number of rounds (r) (Codeluppi et al. 2018; la Manno et al. 2016; Shaffer et al. 2017).

$$n = f \cdot r$$

However, with re-labeling the same RNA molecule over different cycles, it is possible to build a color or binary barcode on the transcript (Figure 6) (Chen et al. 2015; Lubeck et al. 2014). Tracking the signal of a given molecule over the various rounds of labeling would then enable the identification of the molecule by the assigned barcode. This increases the plexity exponentially, so that the number of resolvable targets equals the number of fluorophores to the power of the number cycles performed.

$$n = f^{\,r}$$

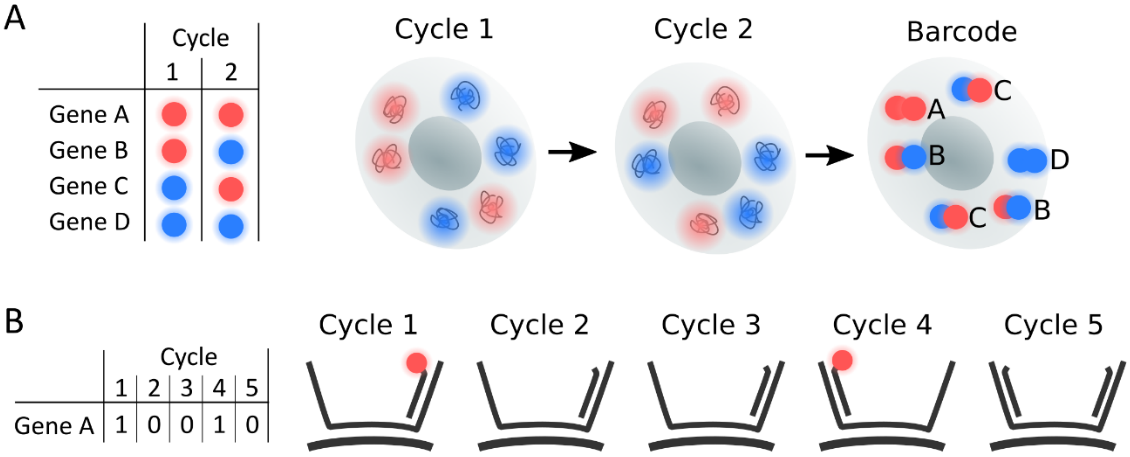

Figure 6 | Barcoded smFISH. A – Dense barcode where two fluorophores are used in two cycles to identify four genes. B - Sparse binary barcode of five bits, where the RNA will light up twice in cycles one and four. The right part shows the encoding probe on the RNA with overhanging tails to which the fluorescent detection probes can bind. Stripping is performed by cleaving the fluorophore off the probe.

2.2.1.2 Barcoding

The barcoded approach could in theory scale the number of targets to the entire transcriptome in only eight rounds of labeling with four fluorophores (4^8 = 65,536) (Lubeck et al. 2014). As a proof of principle for the barcoding strategy, the authors demonstrated the identification of 12 RNA species using four fluorophores and two cycles. To actually scale this method to the full transcriptome a number of technical hurdles had to be overcome.

The most limiting problem for transcriptome wide imaging is the available optical space. The reason is that signal spots corresponding to a single RNA molecule can start to overlap when too many are simultaneously labeled, so that they are not independently identifiable anymore. This is a general problem for smFISH of a single gene because it might underestimate the number of transcripts per cell, but the effect is enlarged with barcoding, because the RNA molecules of multiple genes are simultaneously labeled with the same fluorophore. Furthermore, each additional round adds a chance that this problem occurs to a given molecule, increasing the risk of dropouts in the identification afterwards.

To illustrate; considering that there are on the order of 200,000 RNA molecules in a mammalian cell (Shapiro, Biezuner, and Linnarsson 2013), which all need to be simultaneously and identifiably detected. The distance (d) two molecules minimally need to be spaced to be resolvable is given by the Rayleigh Criterion:

$$d = 0.61\frac{\lambda}{NA}$$

Where NA is the numerical aperture of the microscope objective lens and λ the emission wavelength of the fluorophore. Using the absolute optimal configuration of a lens with a high NA of 1.45 and a blue fluorophore with an emission wavelength of 425 nm, we can see that RNA molecules need to be minimally 220 nm apart to be resolvable by the microscope in XY. The minimal distance in the Z-dimension is many times larger due to the poor resolving power of widefield microscope systems in this axis. smFISH images of a 10 µm section are therefore, usually directly flattened by a maximum intensity projection, so that only the XY-space is relevant for calculating optical density.

Thus, to calculate the minimal optical space required to resolve all 200,000 RNA molecules we can artificially arrange them in a perfect square grid. This would give us a square of 99 x 99 µm, which is many times larger than a typical cell with a diameter 10-15 µm in brain. In addition, RNA transcripts are of course not perfectly arranged, so that the available optical space is even more limited. Simulations show that only several hundred transcripts can be simultaneously detected in the optical space of a cell without overlapping signal causing substantial problems (Borm et al. 2022; Codeluppi et al. 2018).

There are a number of solutions to the optical crowding problem. The most straightforward approach is demonstrated in the seq-FISH method, where only low expressed genes are selected for barcoded detection (Shah, Lubeck, Zhou, et al. 2016). These can still hold information on cell identity and tissue organization, and other genes with higher expression levels can be added as individually labeled genes in non-barcoded rounds. In this study the barcoded genes were encoded with *a dense barcode* where each RNA molecule was labeled in each hybridization round with one of the used fluorophores according to the pre-designed barcoding scheme.

As alternative to dense barcodes, *sparse barcodes* encode the identity of an RNA transcript in a binary code so that the RNA molecule is not labeled every round, making the resulting signal less dense (Chen et al. 2015). Another problem with repeated labeling of the same molecule is the accumulation of errors over the detection cycles. As solution, the authors of this method called MERFISH, implement a barcoding scheme based on Hamming codes, which are error-correcting codes originally developed for telecommunications (Chen et al. 2015). Genes are encoded by barcodes that differ from other barcodes in multiple positions, so that multiple errors need to occur for a barcode to be misidentified. Altogether, this approach increased detection accuracy and allowed for the detection of 1,000 RNA species.

Another solution to optical crowding is the physical expansion of the sample using *expansion microscopy* (Chen et al. 2016; F. Chen, Tillberg, and Boyden 2015). It works by embedding the tissue in a hydrogel that swells when a hypotonic buffer is introduced. This sample preparation method was incorporated in MERFISH so that the optical space is enlarged for barcoding and the errors are reduced at the cost of more imaging (G. Wang, Moffitt, and Zhuang 2018).

Since the initial development of seq-FISH and MERFISH both methods have been further developed, and interestingly, their experimental methods have converged to an almost identical protocol. In both methods the sample, either being cultured cells or a tissue section, is embedded into an acrylamide hydrogel, similar to tissue clearing methods (Chen et al. 2016; Moffitt, Hao, Bambah-Mukku, et al. 2016; Shah, Lubeck, Schwarzkopf, et al. 2016). To encode the barcode, a set of probes are designed which consist of an RNA-binding part and one or two overhanging flaps. These flaps do not hybridize to the RNA but encode the pre-defined barcode for each RNA species (Chen et al. 2015; Eng et al. 2017, 2019). This barcode is subsequently read-out by cyclic fluorescent detection by hybridizing fluorescent probes to the flaps. To remove the signal, previous strategies involved probe digestion by DNase (Lubeck et al. 2014) or fluorophore bleaching (Chen et al. 2015), but currently fluorophores are chemically cleaved off the probe by reducing a thiol linker between probe and fluorophore (Moffitt, Hao, Wang, et al. 2016).

Barcoded smFISH methods, can now reach the detection of up to ten thousand different RNA species (Eng et al. 2019; Xia, Fan, et al. 2019). However, adding more genes does not always add more information, and experiments with a few hundred genes turned out to be a good tradeoff between experimental complexity and biological relevance. Seq-FISH has been applied to study organogenesis in the early mouse embryo (Lohoff et al. 2021). MERFISH has mostly been applied to the brains of mouse and human and enabled comparison between species (Fang et al. 2022; Moffitt et al. 2018; Zhang et al. 2021). These studies could use the highly multiplexed RNA measurements to identify and localize hundreds of cell types in tissue samples to study cellular organization.

2.2.1.3 Linear scaling

Barcoding is a powerful strategy to image a high number of RNA species in biological samples. However, barcoding has some limitations, mostly concerning optical crowding as previously described. Moreover, the alignment of images between rounds is critical and needs to be performed with high accuracy with is technically challenging. The alternative strategy is the linear scaling of targets, where in each round the RNA from a new gene is targeted with a limited set of fluorophores. Although the maximum number of reported targets is only 33 with this strategy, the approach can be sufficient to look at the cell types in a small tissue area or in a targeted manner.

One study used 3 cycles with up to 3 targets per round labeling a total of 8 genes to study radial glia anatomical location and timing in the mouse developing brain (la Manno et al. 2016). This method was later extended to detect 33 RNA species in the adult mouse brain cortex, with which cell types could be identified and the tissue architecture could be studied (Codeluppi et al. 2018). A similar approach has been developed to study up to 19 targets in cultured cells without (Shaffer et al. 2017) and with signal amplification (Dardani et al. 2022; Rouhanifard et al. 2018). Another amplified method performed cyclic smFISH as a post-hock stain on a brain sample for which the synaptic connectivity of a few cells in the sample was first measured using 2-photon microscopy (Nicovich et al. 2019). The combination of a functional measurement such as electrophysiology or calcium imaging, with the post-hock identification of the cell type using cyclic FISH has the potential to unify multiple cellular modalities into the same framework of cell types (Bugeon et al. 2022; Lein, Borm, and Linnarsson 2017).

2.2.2 Sequencing *in situ*

In situ sequencing is an alternative imaging-based strategy to locate a high number of RNA species in tissue samples. Similar to modern sequencing by synthesis, the sequencing of nucleotide targets *in situ* requires the amplification of the target molecule so that the signal is strong enough to be detected. In the various existing *in situ* sequencing strategies this amplification is carried out by rolling circle amplification (RCA) of the to-sequence oligonucleotide, followed by sequencing by ligation or other strategies (Figure 7).

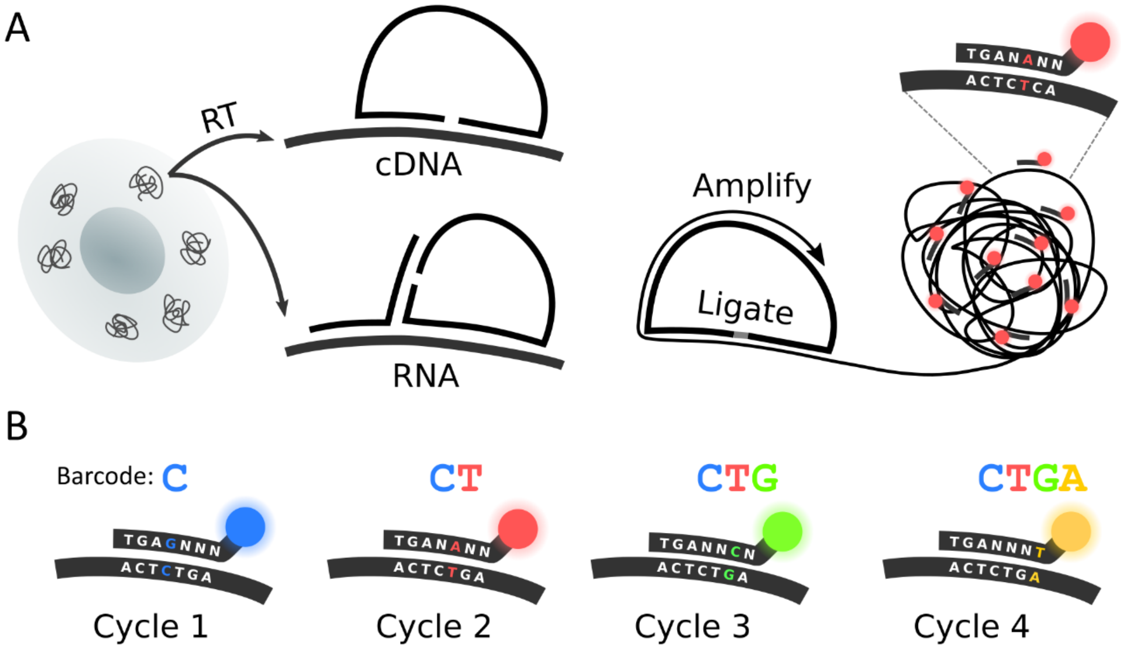

Figure 7 | *in situ* Sequencing. A – Rolling circle amplification (RCA). There are two strategies for RCA, in the first the RNA is reverse transcribed (RT) into cDNA to which the padlock probe is hybridized. The padlock probe can also be hybridized directly to the RNA with a helper probe. The padlock probe is then ligated, and the resulting circular DNA is amplified to generate an RCA product. B – Example of sequencing strategy where in each cycle four probes are introduced for the four bases in a fixed position. The fixed nucleotide is shifted one position each round to retrieve the sequence. Multiple alternative sequencing strategies exist to the one depicted here.

For rolling circle amplification a padlock probe is used. This is a probe with two target binding sites on either end of the oligonucleotide. These both hybridize to the target, so that the 3’ and 5‘ ends of the probe are close together for circularization by ligation. This now circularized probe gets amplified by DNA polymerase, and because it is a circle the polymerase will go round multiple times making many copies of the full sequence of the padlock probe so that there are many binding sites for fluorescent detection probes (Larsson et al. 2010; Nilsson et al. 1994).

Currently there are a number of *in situ* sequencing strategies using RCA that can be split into targeted and untargeted methods.

2.2.2.1 *Targeted sequencing in situ*

For targeted *in situ* sequencing a padlock probe is designed for several different targets. The amplified padlock probe can then be identified by multiple cycles of sequencing by hybridization. This uses 4 fluorescently labeled probes per cycle, that contain an anchor sequence and a stretch of random nucleotides. However, one base in one position is fixed and the four different bases, A, T, C and G are linked to a specific fluorophore. Multiple rounds of hybridizations where the fixed base moves one position away from the anchor allows for sequencing of part of the amplified padlock probe (Ke et al. 2013; Tang et al. 2022).

With this method it is also possible to sequence part of the target to identify single nucleotide mutations (Ke et al. 2013). In this case the two ends of the padlock probe do not meet directly on the target but leave a gap of a few nucleotides between them. This gap can then be filled and ligated to the other end for amplification. So that the specific sequence of the target can be identified, including possible mutations. The authors use both methods to study the spatial distribution of wildtype and mutated transcripts. Since the amplification with RCA can be very strong the signal could also be detected with a smart phone to detect mutations at low cost (Kühnemund et al. 2017).

The first approach which is called *in situ* sequencing (ISS), has further been extended to quantify and locate the transcripts of 99 genes in mouse brain with a specific focus on the organization of the hippocampus (Qian et al. 2020). Additionally, it has been modified to detect 72 targets and applied as post-hock stain after brain tissue was subjected to 2-photon calcium imaging to connect cell type to electrophysiology (Bugeon et al. 2022). Recently, ISS moved away from sequencing by ligation and employed detection by hybridization using a dense color barcode, resulting in better signal to noise ratios (Gyllborg, Langseth, Qian, Choi, et al. 2020). This method has been applied on adult mouse and human brain, and on developing mouse brain to study gene patterning (la Manno et al. 2021).

The above-mentioned methods require as a first step that the target RNA is reverse transcribed into cDNA. Due to the inefficiency of reverse transcription, the sensitivity of RCA based methods is lower than smFISH approaches. The issue with performing RCA directly on the RNA is that the ligating enzyme initially used to circularize the padlock probe could not ligate the DNA on a DNA-RNA duplex. This is now solved by several methods that place the gap of the padlock not on the RNA but in the overhanging tails, which are then brought together by a *splint* probe, so that the ligation takes place on a DNA-DNA duplex (Lee et al. 2022; Lin et al. 2021; Liu et al. 2021; Shi et al. 2022; Sountoulidis et al. 2020; X. Wang et al. 2018). As recent example of this approach, the method STARmap PLUS could locate the transcripts of more than 1,000 genes in 20 sections of the full mouse brain to make an entire spatial atlas at cellular resolution (Shi et al. 2022). By integrating the dataset with a single-cell RNAseq dataset it was also possible to impute the expression levels of 11,844 genes, so that for all those genes a cellular and spatial expression profile could be made.

2.2.2.2 Untargeted sequencing in situ

Targeted methods such as ISS and smFISH have the downside that prior knowledge is needed in order to properly select the targets to include in the assay. As an alternative, it is possible to do de-novo sequencing *in situ*. The first method called FISSEQ used a primer to start reverse transcription on RNA, after which the resulting cDNA was circularized and amplified using RCA. The amplicons were then sequenced using a commercial DNA sequencing kit (Lee et al. 2014, 2015). Resulting reads were indeed untargeted, but this also had the downside that most of the reads came from the abundant ribosomal RNAs (42%). Another issue is that the *in situ* read might be too short to align to the reference genome so that read identification is difficult. In the untargeted variant of Expansion Sequencing, this problem is solved by taking out the targets after *in situ* sequencing for longer reads next-generation sequencing. (Alon et al. 2021). Furthermore, in this method, *in situ* sequencing is combined with expansion microscopy so that transcripts can be localized to nano-scale structures such as dendrites and axons of neurons.

2.2.3 Signal amplification

Traditional smFISH signal is generated only by a few fluorophores bound to the target. For instance, in the widely used smFISH protocol by Raj *et al*. 2008 a maximum of 48 individually labeled probes bind to an RNA molecule. This generates a diffraction limited signal spot that can be detected. However, due to the low number of fluorophores per target, a microscope with an objective lens with a high Numerical Aperture, a sensitive camera and long exposure times are needed. This limits both the imaging throughput to maximally a few square millimeters and generates a barrier for labs to set up the method.

The previously described *in situ* sequencing methods solve this problem by amplifying the target using RCA, so that many more fluorophores can bind to a target. The resulting signal can be detected by lower power objectives and larger areas of roughly one square centimeter can be imaged in reasonable time.

Apart from RCA there are many other signal amplification strategies (Figure 8). However, the problem with amplification is that there is a large risk of generating false positives. In smFISH with typically 48 singly labeled probes, it is difficult to generate these errors because a single probe is not detectable and only when sufficient probes accumulate on the target RNA a signal is generated. Single probes that bind non-specifically to a random sequence or get otherwise stuck in the tissue, are simply not strong enough to generate a signal on their own. In contrast, amplification methods usually build a linearly amplifying or branching fluorescent structure from a starting probe. If one of these initiators would bind non-specifically it could start this amplification process and generate a false positive. Therefore, amplification needs to happen in stringent conditions with strong washes to remove any unbound initiators.

In branched DNA FISH a tree of oligonucleotide probes is built upon the RNA target to increase the number of binding sites for fluorescent probes on the target (Kern et al. 1996; Player et al. 2001; Xia, Babcock, et al. 2019). In a related strategy called SABER FISH, a branching structure is built upon the target the doubles the number of fluorescent probe binding sites per amplification cycle (Kishi et al. 2018). Similarly, ClampFISH builds an exponentially growing tree of padlock probes that are crosslinked by click-chemistry for each round of amplification (Dardani et al. 2022; Rouhanifard et al. 2018). Padlock probes are also used in hybrid RCA – smFISH methods, where probes with an overhanging tail are first hybridized to the RNA target, to which then the padlock probes are subsequently hybridized, followed by RCA (Sountoulidis et al. 2020; Wu et al. 2018). Lastly, in Hybridization Chain Reaction (HCR) initiators are bound to the target RNA with two overhanging flaps. Then hairpins probes are introduced which are self-complementary that only open up when they bind to the initiator sequence. The newly opened up sequence is in turn able to hybridize and open another hairpin. The hairpins themselves can be fluorescently labeled or fluorescent probes can be hybridized to them in multiple cycles (Choi et al. 2016, 2014; Dirks and Pierce 2004; Shah, Lubeck, Schwarzkopf, et al. 2016; Wang et al. 2021).

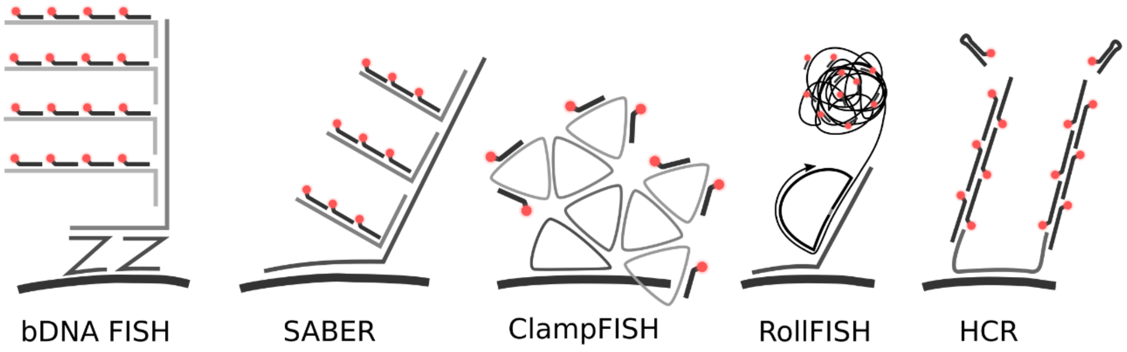

Figure 8 | Amplification strategies for smFISH. From left to right: branched DNA FISH, Signal Amplification by Exchange Reaction (SABER), ClampFISH, RollFISH and Hybridization Chain Reaction.

Because an amplified signal is detectable using lower power microscope objectives with a longer working distance than high power objectives, RNA can also be detected at depth so that RNA localization can be studied in 3D. This furthermore requires that the tissue sample is optically cleared, to prevent the signal from scattering an opaque tissue sample, but then cells can be studied in their original tissue context and not just a thin 10 µm section that is typically used (Shah, Lubeck, Schwarzkopf, et al. 2016; Wang et al. 2021; X. Wang et al. 2018).

2.2.4 Human adult samples

A few of the described methods so far are compatible with human brain samples. However, using microscopy on human samples is not trivial due to the presence of lipofuscin in aged human samples. These are lysosome-like granules containing lipoprotein aggregates that are extremely auto-fluorescent so that it is difficult to pick up the weak smFISH signal and it can generate false positives. Many strategies have been tried to dissolve or otherwise remove lipofuscin but to no avail. Furthermore, gel embedding of the tissue and protein digestion leaves the lipofuscin virtually intact. The oldest strategy is quenching the autofluorescence by quenchers such as copper, Sudan Black or True Black (Schnell, Staines, and Wessendorf 1999) , and these work well for the amplified signal of RCA based ISS (Gyllborg, Langseth, Qian, Salas, et al. 2020). However, these do not work as well for unamplified smFISH because these quenchers also quench the fluorescence of the smFISH signal, dropping them below detection limit. Another strategy is to bleach the lipofuscin with light before imaging (Fang et al. 2022; Sun and Chakrabartty 2016). This is a simple and effective strategy, but it requires up to one day in a setup where the tissue sample is cooled so that heat from the light does not degrade the RNA (Park et al. 2022). The last strategy involves complete removal to the tissue sample after the RNA has been transferred to a substrate for imaging (Borm et al. 2022). Nevertheless, working with human samples is difficult due to rare availability and long post-mortem intervals, but these approaches pave the way for the making of a human cell atlas (Regev et al. 2018).

2.2.5 Probe selection

All targeted *in situ* RNA localization methods require that the genes are decided beforehand. In the initial development of these methods, the genes were sometimes picked based on the technical requirements of the methods, such as low expressed genes (Shah, Lubeck, Zhou, et al. 2016) or long transcripts that could fit a large number of barcode-encoding probes (K. H. Chen et al. 2015). Nowadays, gene targets are picked to be biologically relevant in order to allow for cell type identification or other goals. These choices heavily rely on previous knowledge and are currently commonly based on existing single cell RNA-seq datasets of the same tissue (Codeluppi et al. 2018; Moffitt et al. 2018). Initially genes were selected manually (Codeluppi et al. 2018), but common methods nowadays are to use differential gene expression or mutual information analysis on single cell RNA-seq clusters to inform gene selection (Moffitt et al. 2018; Zhang et al. 2021). Furthermore, there are now more advanced tools available based on Principle Component Analysis (PCA) (Kuemmerle et al. 2022), random forest classifier (Aevermann et al. 2021) or deep learning (Covert et al. 2022) to select genes. Moreover, the first mentioned method also provides a full pipeline for probe sequence design and provides rules for cell type identification with the chosen genes (Kuemmerle et al. 2022).

After gene selection, probe sequence design is another critical step for the targeted methods. Probes need to be specific, meaning that they will not recognize other similar transcripts, and have uniform hybridization characteristics as all other probes, meaning that the melting temperature of all probes is roughly the same. The melting temperature of a probe is the temperature at which 50 percent of bound probes will de-hybridize and is thus a measure of hybridization strength. If the hybridization conditions, determined by the temperature, salt concentration and the concentration of destabilizing agents such as formamide or ethylene carbonate, are too permissive, probes will bind off-target. Conversely, if the conditions are too stringent the probes will not be able to hybridize. The conditions have the be just right to allow the correct probes to bind, but prevent aspecific binding, and therefore, it is important that all probes have a similar melting temperature so that they can all be simultaneously controlled by controlling the hybridization conditions.

To help design these thousands of probes there are a number of tools available that take these constraints into consideration to generate the probe sequences (Beliveau et al. 2018; K. H. Chen et al. 2015; Hershberg et al. 2021; Kuemmerle et al. 2022; Tsanov et al. 2016). These sequences can then be appended with barcode encoding tails, padlock backbones or they can be directly labeled with a fluorophore.

2.2.6 Cell assignment

After the RNA molecules are labeled and imaged, the signals are called using peak detection in image analysis, and optionally barcodes or sequences are decoded. This results in a collection of RNA localizations in space with their gene or sequence label. These RNA molecules can be assigned to individual cells to make a cell by gene matrix, similar to the output of single cell RNA-seq, but with the addition of the spatial location of every cell. To assign transcripts to cells there are several strategies.

The most common strategy is to label either the cell body, cell membrane and/or nucleus alongside the RNA labeling experiment, which can be segmented into individual cells (Buxbaum et al. 2015; Codeluppi et al. 2018; Lohoff et al. 2021; Shah, Lubeck, Zhou, et al. 2016). The RNA molecules can then be assigned to cells if they fall within the perimeter of the cell. This approach relies on cell segmentation, which can be challenging due to high densities of cells, making it difficult to manually segment them. More modern approaches use machine learning to perform segmentation, but these are also limited because of the difficulty of generating ground-truth data to base the learning on. Nevertheless, recent years has seen a large improvement of computational segmentation methods especially because of the implementation of neural networks, which are now routinely applied to large datasets (Berg et al. 2019; He et al. 2015; Stringer et al. 2021; Weigert et al. 2019).

The proper choice of staining and segmentation approach also depends on the tissue type that is studied. In most tissues the cells tesselate the three-dimensional volume similar to how the bubbles of a foam are organized and closely resembles a Voronoi tessellation (Gómez-Gálvez et al. 2021). For these situations, a cell segmentation approach where the membranes are labeled, best captures the cell morphology to assign RNA molecules to cells (Lohoff et al. 2021). However, cells in other tissues, such as in the brain, have much more complex morphologies. The dendrites and axons of neurons for instance can span micrometers to meters in length and these are generally much thinner than the resolution limit of regular microscopes, so that they cannot be properly segmented. To segment brain cells one would need to increase the resolution, either by electron microscopy (Kasthuri et al. 2015; Zheng et al. 2018), expansion microscopy (Alon et al. 2021; Chen et al. 2016; F. Chen et al. 2015) or super resolution microscopy (Hell et al. 2015). However, these methods are not always compatible with the RNA labeling experiment and come with additional experimental constraints. For the brain another strategy is to label the cell soma that contains the bulk of the transcriptome by targeting the poly-A tails of mRNA using a FISH probe, as a surrogate for the cell volume (Buxbaum et al. 2015; Codeluppi et al. 2018; Fang et al. 2022).

If enough transcripts are labeled inside a cell this can also be used for cell soma segmentation directly (Chen et al. 2017; Sun et al. 2020). Related to this approach, the RNA molecules can also be segmented into cells directly by analyzing which transcripts are likely found in the same cell, by clustering transcripts based on their identity and proximity (He et al. 2021; Petukhov et al. 2021). Harnessing the expression profiles found by single-cell RNA seq, transcripts can moreover be assigned to segmented nuclei in a probabilistic manner, to aid the difficult task of assigning molecules in the brain where cell morphologies can be highly intermingled (Qian et al. 2020).

Alternatively, the segmentation step can also be skipped entirely, by directly analyzing the RNA localization to find cell type signatures of co-localizing transcripts (Park et al. 2021). Another, segmentation free approach, leverages graph theory to embed RNA localizations in a graph to study principles of RNA topology (Partel and Wählby 2021). These approaches circumvent the difficult and often error-prone cell segmentation step and enable the exploration of spatial transcriptome organization.

2.2.7 Microscopy methods conclusion

The imaging-based methods are characterized by high resolution imaging which allows for single cell segmentation. Typically, the gene throughput is limited to tens or hundreds of targets but there are demonstrations where multiple thousands of genes are detected. This throughput is enough to make maps of cell types in target tissues and this group of methods is promising for making highly detailed spatial maps of cells and their types or state.

2.3 Sequencing

In parallel to the imaging approaches, next generation sequencing approaches have been developed. These nearly all rely on the tagging of transcripts with a spatial barcode so that the RNA identity and location can be resolved using DNA sequencing. These methods generally have the advantage of being transcriptome wide and untargeted. Furthermore, by relying on highly optimized DNA sequencing pipelines they can quickly be implemented by many users and do not require the building of complex setups that are used for the imaging approaches.

However, interestingly, these imaging setups are surprisingly similar to a sequencing machine. Both image a flat surface at high throughput for multiple cycles and a fluidics system dispenses reagents to read out the next bit, letter or gene (Borm 2022; Moffitt and

Zhuang 2016). In fact, people are now converting old sequencing machines to work for cyclic imaging, since these sequencers are perfectly engineered and optimized for these kinds of tasks (Pandit et al. 2022). Nevertheless, the fact that DNA sequencing machines are a few years ahead in their development and many companies and facilities have set up streamlined pipelines, makes the spatial sequencing approaches easy to implement and therefore widely adapted.

2.3.1 Patterned surface

The first group of methods relies on a surface that is micropatterned with spatial barcodes in known positions, coupled to a poly-T sequence that can hybridize to the poly-A tail of mRNA. A tissue section is then placed on this patterned surface and the RNA is allowed to diffuse to the nearest barcodes where they get captured (Figure 8). Sequencing of both the barcode and RNA will then tell the location of the transcript.

2.3.1.1 Microarray

The method that invented this principle is called *Spatial Transcriptomics*, which uses a microarray of spatial barcodes (Figure 8, left) (Stahl et al. 2016). Microarrays were the main technology to study gene expression before it was replaced by sequencing, and consists of an array of spots, each with a unique DNA sequence, that are immobilized on a surface. The Spatial Transcriptomics method adapted this format to make an array of spatial barcodes where each barcode has a known location and an RNA capture tail. A tissue section is then placed on top, and permeabilized so that the RNA can diffuse to the spots where they get captured. Reverse transcription is performed with the capture probe as primer to incorporate the spatial barcode and RNA sequence into the cDNA. Then the tissue sample is digested, and the cDNA is released for sequencing so that the spatial barcode can be identified along with the RNA identity. The barcode will then tell approximately where the RNA originated from in the tissue, so that the spatial transcriptome of the sample can be studied.

Initially the method had a spot size of 100 µm with an inter-spot distance of 200 µm in a rectangular pattern. Since the development in academic setting, the method has been commercialized and is now available under the name Visium by the company 10X Genomics and is widely used (Moses and Pachter 2022). The spot size has now been reduced to 55 µm with a distance of 100 µm between spots that are arranged in a hexagonal grid for tighter packing. These spots are still large and can contain the signal of multiple cells. To increase the resolution a number of methods have been developed that are discussed in the sections below. Alternatively, it is possible to deconvolve the contribution of multiple cells, to the signal coming from one spot with the help of reference single cell RNA-seq data (Biancalani et al. 2021; Cable et al. 2022; Kleshchevnikov et al. 2022).

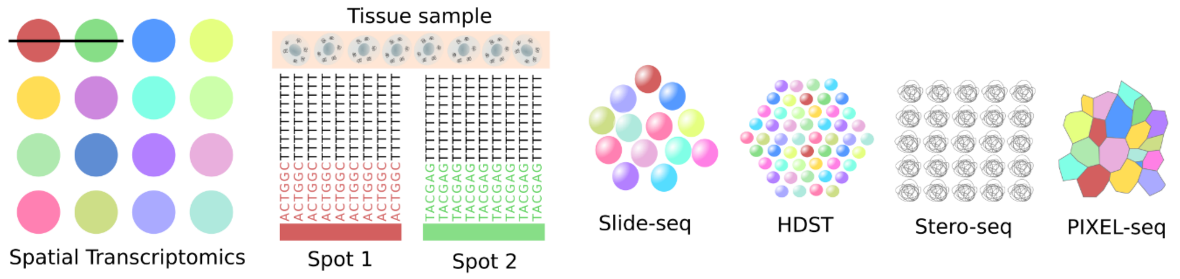

Figure 8 | Spatial RNA sequencing technologies. In Spatial Transcriptomics depicted on the left a tissue section is placed onto microarray of uniquely barcoded spots. The RNA diffuses out of the tissue and binds to the poly-T tail of the spatial barcodes. On the right four variations on the array are depicted: Slide-seq uses 10 µm barcoded beads, HDST 2 µm beads, Stereo-seq uses 200 nm DNA nanoballs and PIXEL-seq uses bridge-PCR products with an average size of 600 nm.

Spatial Transcriptomics has been applied to many different tissue types such as mouse brain (Ortiz et al. 2020; Ortiz, Carle acute n, and Meletis 2021; Stahl et al. 2016), developing heart (Asp et al. 2019), intestine (Parigi et al. 2022), liver (Yu et al. 2022), and many types of cancer (Maniatis, Petrescu, and Phatnani 2021), among many other tissues of mouse and human (Moses and Pachter 2022). Furthermore, high throughput pipelines have been set up that can process 64 sections in 2 days for large spatial screens (Vickovic et al. 2022). In contrast to the imaging approaches the clear advantage of the sequencing approach is that it is untargeted, so that the data can be used exploratory for the discovery of genes that are spatially differentially expressed, or to find regions in the tissue that share similar transcriptional profiles (Kats, Vento-Tormo, and Stegle 2022; Ortiz et al. 2020; Svensson, Teichmann, and Stegle 2018).

2.3.1.2 Patterned beads

To increase the resolution of this approach the microarray can be replaced by a surface covered with barcoded beads (Figure 8, right). In one approach, called Slide-seq, the beads are the same beads as used for droplet based single cell RNA-seq (Rodriques et al. 2019; Stickels et al. 2021). These beads are individually barcoded with barcoded RNA capture oligos and deposited on the surface. However, the deposition is random and not deterministic like the pre-defined locations of every barcode on the Spatial Transcriptomics microarray. Therefore, the spatial location of each bead, and thereby barcode, first needs to be determined using a cyclic FISH imaging approach that labels each bead with a unique color code over the cycles to identify the spatial barcode later used for sequencing. Once this is done a tissue section can be placed on the beads to transfer the RNA, followed by tissue digestion and preparation of the sequencing library. Furthermore, it is worth noting that the beads on the surface are more densely packed than the spots on a microarray, but they do not form a perfect grid, so that there is an occasional gap. The beads have a diameter of 10 µm which is around the size of a cell soma and therefore approaches single cell resolution. Nevertheless, with these methods it is hard to determine if a single cell contributed to one or multiple beads, due to lateral diffusion of the RNA or the possibility that two beads share part of the cell area.

To control the bead spacing and placement on the surface it is possible to deposit beads with a unique barcode on an array of small wells (Vickovic et al. 2019). This approach called *High-Definition Spatial Transcriptomics* (HDST) uses beads that are just 2 µm in diameter (Figure 8). Using a reference image of the tissue stained with hematoxylin and eosin taken before the RNA is transferred, sequenced transcripts could be assigned to individual cells, so that the organization of cell types in a tissue could be studied.

2.3.1.3 Spatial Transcriptomics expansion

Another way to increase the resolution of Spatial Transcriptomics is to enlarge the sample, rather than making the capture features smaller. Similar to the discussed approaches for the imaging-based methods, this method embeds the sample in a swellable gel using the Expansion Microscopy method (F. Chen et al. 2015; Fan et al. 2022). After sample expansion the gel is placed on a Spatial Transcriptomics slide and the RNA is transferred. This increases the resolution from the standards spot size of 55 µm to approximately 20 µm in the original tissue coordinate space. Furthermore, the authors show that also the RNA transfer efficiency is improved.

2.3.1.4 DNA nanoballs

The highest resolution methods for array based spatial methods is Stereo-seq, with a spot size of 220 nm and an inter-spot distance of 500 or 715 nm on a square grid (Figure 8) (A. Chen et al. 2022). The spots are DNA nanoballs that are created by rolling circle amplification (RCA) and deposited on a patterned surface. This surface is the same as is used for the sequencing machine by the company called MGI. This sequencer is subsequently also used to determine which barcode ended up in which location. The RCA products contain a 22-nucleotide poly-T stretch and a spatial barcode so that RNA can be captured and located in space.

Using an image segmentation approach on images of cell nuclei taken before RNA capture allowed the segmentation of individual cells so that the cell type could be determined, and their spatial distribution studied. The area that can be studied with Stereo-seq is roughly a square centimeter and the authors used this to study mouse embryonic development sampling a few sagittal sections of every day of development between 9.5- and 16.5-days post conception. This gave insight into organ development and cell fate specification.

2.3.1.5 Bridge PCR

The last group of methods in this category relies on bridge-PCR to form areas or volumes of barcoded capture probes. In the first step barcoded templates are sparsely seeded onto a surface of small attachment oligos. The templates have sequences on either end that can hybridize to these attachment oligos, so that when multiple cycles of PCR are performed the copies of the template will *walk* over the surface, every time attaching to a new nearby attachment oligo until the area is filled with identical copies of the template. This results in a surface that is filled with spatially restricted *polonies* of the barcoded capture oligo.

The first method called Seq-scope uses repurposed commercial surfaces made for the Illumina sequencer flow cell that is optimized for the bridge-PCR procedure (Cho et al. 2021). This results in a dense lawn of polonies with a center-to-center distance of 600 nm on average, so that multiple polonies cover the area of one cell. In the second method, PIXEL-seq, the bride-PCR step is performed in a thin acrylamide gel, resulting in a gel filled with polonies with an average size of 600 nm (Figure 8) (Fu et al. 2022). Again, combined with image-based segmentation these methods can reach single cell resolution.

2.3.2 Spatial barcode tissue

The opposite approach is also possible, where not RNA molecules are moving towards a spatial barcode, but where the spatial barcode is moving towards the transcripts (Figure 9). One approach called DBiT-seq uses a microfluidic device that can flow spatial barcodes over the tissue in small channels (Liu et al. 2020). These then diffuse into the tissue where they hybridize to the RNA and function as primers for reverse transcription, generating up to 50 barcoded rows in the tissue. Then the device is rotated 90 degrees and a second set of 50 barcodes is flown through the channels which ligate to the first barcode. This creates a grid of squares in the tissue where each grid is uniquely labeled with a combination of a row and column barcode. After amplification, sequencing is used to locate the detected molecules in space. The largest channel configuration is 50 µm wide and the maximum resolution that can be reached is with a channel size of 10 µm wide. Current implementations of the microfluidic device always have 50 channels generating a grid of 2,500 tiles where there is a tradeoff between resolution determined by the channel size and spatial coverage. One downside of this method is that the channels need to be separated by a wall so that there is no data between rows and columns and the spatial data is not continuous. However, the approach requires only a simple setup and other modalities can be co-detected in the same assay. In the original publication the authors co-detect up to 100 proteins next to the transcriptome and recently other sequencing-based methods such as CUT&Tag to detect the genome location of histone modifications, gained a spatial dimension (Deng et al. 2022; Liu et al. 2020).

Another approach called sci-Space, uses an array of barcodes deposited onto a glass surface (Figure 9). This array is then flipped onto a tissue section, after which the spatial barcodes diffuse into the tissue and tag the nuclei of cells. The tissue section is then scraped off from the slide and the nuclei are sequenced with an existing single nuclei RNA-seq method (Sanjay R Srivatsan et al. 2021). This gives a dataset where the whole transcriptome is sequenced and has inherently single nuclei resolution, but some of the nuclei that were under the barcoded spot can also be located in space. The spots have an average diameter of 73 µm and an inter-spot distance of 222 µm in a rectangular grid. The method is quick and nicely taps into highly optimized single cell RNA-seq pipelines, but a downside is that these pipelines can only capture a fraction of the cells in the original section, so that the spatial localizations are sparse. Nevertheless, the area that can be covered scales easily and sections of the full mouse embryo at embryonic day 14.5 were studied.

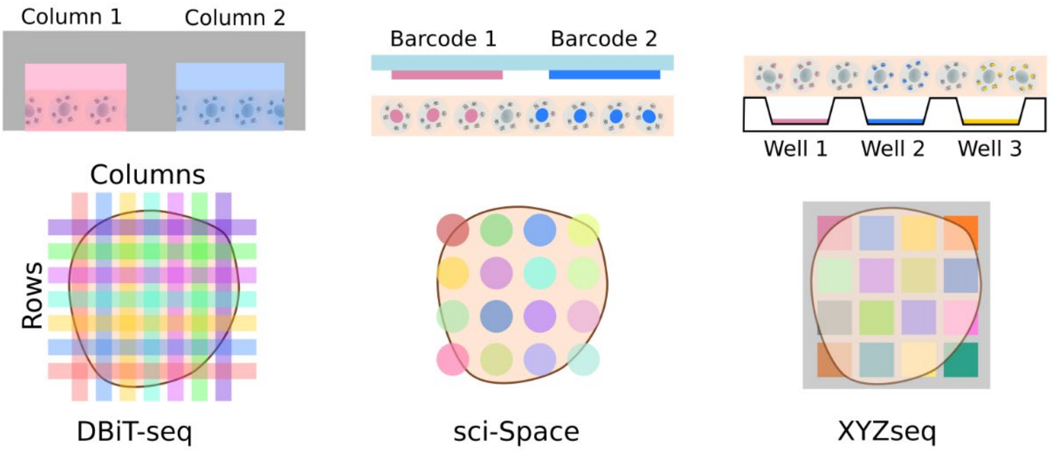

Figure 9 | In-tissue spatial barcoding. In DBiT-seq spatial row a column barcodes are introduced to the tissue using a microfluidic device that combinatorically tags the RNA and proteins in the intersections. Sci-Space uses a microarray of spatial barcodes that diffuse into the tissue and label the nuclei of proximate cells. ZYXseq places the tissue on a microwell array where each well is tagged with a unique spatial barcode that labels the RNA of the cells in the well. Drawings are not too scale and, the respective strips/spots/wells are larger compared to the depicted cells in reality.

A similar method called XYZseq uses a microwell array with wells of 500 µm in diameter, that each contain a spatial barcode. After placing a tissue section on top these then tag the RNA in the tissue above it (Figure 9) (Lee et al. 2021). The cells in the section are subsequently dissociated and distributed into another microwell array to add a second tag to the RNA, so that the RNA has a spatial tag and cell tag. Together they form a combinatorial barcode that can identify transcripts from individual single cells which can be located in space with a resolution of 500 µm.

2.3.3 ROI selection

One of the earlies transcriptome wide spatial methods was laser capture microdissection coupled with RNA sequencing (For a review see: Moses and Pachter 2022). In this approach a tissue section is placed on a membrane and imaged by a microscope. A Region of Interest (ROI) is then selected based on the image and this area is cut out with a laser and caught in a tube for sequencing. This is very powerful for targeted spatial questions. However, if we would draw the parallel that one ROI in these methods is the same as one spot/bead in the sequencing-based methods, the number of ROIs is much smaller. However, it allows for more flexibility where the ROI is not bound by a certain shape or grid, so that it can be tuned to the question at hand.

The laser capture microdissection principle has since inspired other methods that are related to spatially resolved transcriptomics, but instead of physically removing a piece of tissue, local illumination is used to tag or release RNA from a specific anatomical region. The first method, TIVA, uses a caged capture probe that gets activated (uncaged) after light stimulation so that it can hybridize RNA in the illuminated area. The capture probe contains a biotin that can then be used for isolation and sequencing. This approach is even applicable to living cells, but it can only target a single ROI per sample. However, the company Nanostring has used a similar approach that is multiplexable. In their method, Digital Spatial Profiling, probes are targeted to up to 18,000 genes and 44 proteins, which each contain a photocleavable identifier (Merritt et al. 2020). After ROI selection the area is illuminated to release these identifiers which are then sucked up with a small pipette and put in a well of 96-wells plate. This process can be repeated multiple times to select different areas of the tissue and has for instance been used to study multiple layers of the developing human brain cortex (Roberts et al. 2021).

Another way to multiplex ROI sequencing is to use multiple rounds of *in situ* tagging of transcripts or cells. These methods use a deformable mirror array, which are for instance used for image projectors, to pattern the tissue with light. Zip-seq uses uncagable antibodies that tag living cells in a spatially defined way after illumination and which can later be recovered using single cell RNA-seq (Hu et al. 2020). Multiple areas are targeted by multiple rounds of patterning with differently barcoded antibodies. Similarly, Light-seq also uses multiple rounds of tagging but the photoactivatable probes tag cDNA in a cell culture or tissue sample after which they are released for sequencing while keeping the sample intact (Kishi et al. 2022). The intact sample can then be used for protein stains or other microscopy assays.

These methods are maybe slightly orthogonal to the methods previously discussed as they are meant for answering a specific research question that guides the ROI selection, rather than the broad discovery approach. Possibly they are also more biased because of this, but flexibility and possibility for TIVA and Zip-seq to be applied on living cells opens unique possibilities for discovery.

2.3.4 Sectioning and tomography

For highly stereotyped tissues such as drosophila or zebrafish embryos it is also possible to study spatial transcriptome variations by cryosectioning the full sample (Figure 10, left). Sequencing the RNA from each individual sections will then reveal transcriptome variations along the sectioning axis (Combs and Eisen 2013; Junker et al. 2014). These methods were used to study anterior-posterior patterning by sectioning the embryos along this axis. Furthermore, this method has also been applied to study patterning in gastruloids, which are *in vitro* grown three-dimensional embryo-like cultures, reaching a resolution of 8 µm (van den Brink et al. 2020).

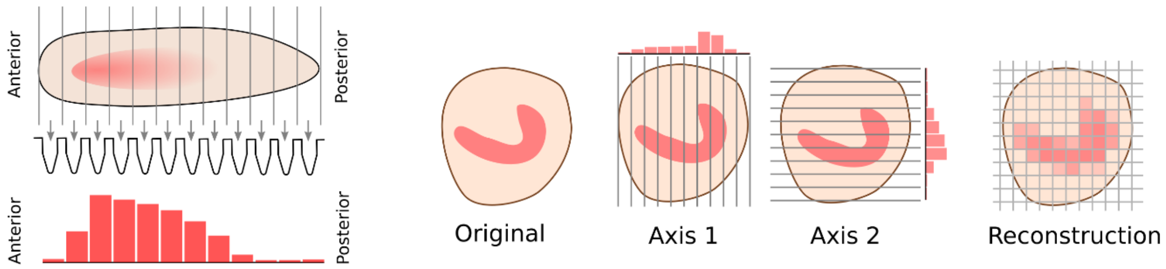

Figure 10 | Spatial reconstruction of gene expression by sectioning. On the left, the anterior-posterior pattern of gene expression depicted in red can be recovered by sectioning and quantification of each slice. On the right, this can be extended with tomography to reconstruct gene expression in multiple dimensions.

It is also possible to extend this principle to reconstruct gene expression in multiple dimensions, instead of just one (Figure 10, right). If one could section the same tissue multiple times in all three axes it would be possible to locate gene expression in three dimensions. Dicing the tissue in this way is unfortunately technically very challenging and would require the sequencing of many *voxels*. To circumvent this problem, two methods use the fact one sample could be considered identical to other samples if they are highly stereotyped. In the first method the brains of inbred mouse strains are considered identical, so that three brains are sectioned in all three different axis and the transcriptome is measured by microarray. This data could then be used to reconstruct gene expression in three dimensions with a resolution of 5 µm using tomography (Okamura-Oho et al. 2012). However, tomography algorithms fail for complex spatial structures if only three axes are measured. STRP-seq solves this by adding more cutting angles (Schede et al. 2021). In this method, adjacent sections are used as replicates and cut into strips at various angles, to reconstruct gene expression of the mouse brain in two dimensions. Furthermore, it was used to map gene expression in the lizard brain, demonstrating the power of sequencing to map gene expression in species without much prior knowledge.

2.3.5 Imaging-by-sequencing

Imaging-by-sequencing is a completely different approach where DNA sequencing is used to determine how molecules are related to each other in space. Consider the bridge-PCR methods that were discussed before. In those methods, spatial barcodes are sparsely seeded on a surface, after which they are amplified by bridge-PCR. The PCR products will grow on this surface radially outwards from the point where the first spatial barcode was bound. The amplification will continue until the products reach an adjacent amplification product and there are no more binding sequences left on the surface. For Seq-Scope and PIXEL-seq (see section 2.3.1.4) this is the end point of the creation of the patterned surface (Cho et al. 2021; Fu et al. 2021).

Imaging-by-sequencing approaches however, go one step further by using special barcoded oligonucleotides that can recombine with neighboring barcodes when they are in close proximity. As an example, let us consider that barcode *A* is located next to barcode *B* in space. At the interface they will combine to generate a sequence of *AB*. Sequencing all oligonucleotides will give us sequences of *A*, *B* and *AB*, so that we can deduce that barcode *A* should be located next to barcode *B* in space. Extending this framework of pairwise constraints enables the reconstruction of the spatial layout in two dimensions without the need to make a reference image or any other way of anchoring the sequences (Hoffecker et al. 2019). It can even be extended to three dimensions if molecules can diffuse in a matrix instead of *walking* over a two-dimensional surface (Fernandez Bonet and Hoffecker 2022). The next trick is to incorporate useful information into the sequences so that any target information could be incorporated and later spatially reconstructed. One demonstration of this approach used this principle to locate transcripts of a few genes in space with cellular resolution (Weinstein, Regev, and Zhang 2019). A larger scale demonstration with multiple genes in tissue samples has to be performed still, but it is an interesting orthogonal approach. However, reconstructing large spaces and multiple genes would require a large amount of sequencing.

2.3.6 Spatial reconstruction

The last group of methods would actually fall in both the imaging and sequencing categories, since these methods take single cell RNA-seq data and combine it with spatial expression profiles to compute the likely location of the cells in the reference image. If the tissue contains a number of genes that are expressed in orthogonal gradients or distinct regions, this knowledge can be used as a way to decide where a cell is likely located based on its expression level of these genes (Achim et al. 2015; Halpern et al. 2017; Karaiskos et al. 2017; Moor et al. 2018; Satija et al. 2015; Wei et al. 2022). Instead of individual cells, the likely location of cell types can also be defined, which furthermore, allows for the spatial imputation of genes that were not measured in the spatial assay (Abdelaal et al. 2020; Biancalani et al. 2021; Stuart et al. 2019; Welch et al. 2019; Zeisel et al. 2018). In a sense these approaches combine the spatial resolution of the imaging methods with the high gene throughput of the sequencing methods in a best-of-both-worlds scenario. Nevertheless, the reconstruction only works well if the sequenced gene correlates well with the genes measured in the spatial assay, and benefits from tissues that have clear spatial patterns. Furthermore, it is not possible to look at cell-cell interactions because there is no guarantee that if two cells are placed next to each other computationally, they were also next to each other in the original tissue.

2.4 Technical tradeoffs

As described, there are a large number of methods to measure the transcriptome in its spatial context and the field is growing fast (Crosetto, Bienko, and Oudenaarden 2014; Lein et al. 2017; Moses and Pachter 2022). Each approach has its own advantages and disadvantages, and there is no clear best method at this point. The ideal method to learn about the functioning of single cells in the tissue would be characterized by cellular or subcellular resolution, full transcriptome coverage at high sensitivity, high spatial throughput and easy implementation. In this section, I will discuss how the various methods compare on each of these items. To simplify the comparison, I will not consider methods that cannot get single cell resolution in tissues (tomography and reconstruction) where cell-to-cell distances cannot be studied. Nor the immature imaging-by-sequencing approach and the ROI-selection technologies because these do not scale to many cells/areas like the other methods do.

2.4.1 Resolution

In terms of resolution the smFISH based methods have the highest resolution which is only limited by the diffraction limit of light at approximately 200 nm (K. H. Chen et al. 2015; Codeluppi et al. 2018; Eng et al. 2019). This can even be increased by expansion microscopy to reach a resolution of 70 nm (Chen et al. 2016). With an approximate cell diameter of 10-20 µm this resolution is more than enough to assign detected RNA molecules to cells and even study the subcellular organization of the transcriptome (Battich et al. 2013; Xia, Fan, et al. 2019).

Microscopy methods relying on rolling circle amplification on the other hand, are limited in their resolution by the size of the amplification products, which are roughly 1 µm in diameter (Deng et al. 2018; Ke et al. 2013). This is large and it is estimated that a maximum of 90 would fit inside a typical cell, resulting in a physical crowding problem rather than optical crowding (Ke et al. 2013). It is however possible to shrink the RCA products to a smaller size after they are made, but the question is if the RCA is not already inhibited by physical crowding when they are produced (Clausson et al. 2015).

For the sequencing-based methods (both the patterned surface and spatial in-tissue barcode), the resolution is determined by two factors. The first is the feature size that determines the granularity by which a detected molecule can be placed in space. The second factor is the lateral diffusion of the RNA molecules. These methods rely on RNA molecules or spatial barcodes to diffuse to a surface or target respectively, but these can also diffuse laterally, reducing the resolution. Unfortunately, the length scales of this lateral diffusion process have not been accurately determined experimentally. Initially, when these methods had large detections spots of 100 µm with a large space between spots, the effect of lateral diffusion was likely negligible compared to the spot size (Stahl et al. 2016). Now that the spots are reduced to 200 nm the effect of lateral diffusion is likely more pronounced and although this method demonstrated a granularity that is much smaller than a cell it remains a question if only a single cell contributed to the signal on each feature (A. Chen et al. 2022).

2.4.2 Spatial throughput

For microscopy methods the direct tradeoff with resolution is spatial throughput. To detect the smFISH signal it needs to be imaged at high resolution using high power microscope objectives. These have a high magnification factor which also reduces the size of the field-of-view. Furthermore, the objectives also need to have a high numerical aperture, which has the effect that the depth-of-field of the lens is very shallow. The dept-of-field determines how thick the area is that the objective can see sharp. For high numerical aperture objectives this is typically just a few hundred nanometers, so that multiple images need to be taken in the depth dimension (Z) to capture all the information. As example with a dept-of-field of 300 nm and a tissue section of 10 µm, at least 34 images need to be taken in a Z-stack, which takes a lot of time. Especially since the sample needs to be imaged multiple times for each cycle, so that experiments can take multiple days to weeks to image a few mm^2 (Codeluppi et al. 2018).

Furthermore, unamplified smFISH approaches typically have a low number of fluorophores on the target so that longer exposure times are needed with expensive high-sensitive cameras. Amplifying the signal with hybridization chain reaction, branched DNA FISH or other methods reduces the exposure time at the cost of a longer labeling protocol (Choi et al. 2016, 2014; Dardani et al. 2022; Dirks and Pierce 2004; Kern et al. 1996; Kishi et al. 2019; Player et al. 2001; Rouhanifard et al. 2018; Shah, Lubeck, Schwarzkopf, et al. 2016; Wang et al. 2021; Xia, Babcock, et al. 2019). However, they make imaging faster and still generate structures that are smaller than the diffraction limit so that the spot size is not increased by amplification and the issue with optical space is not aggravated (Xia, Babcock, et al. 2019). Nevertheless, modern cameras allow for short exposure times of roughly 100 milliseconds for unamplified smFISH, so that mechanical movement and filter changes dominate the total imaging time. Shorter exposure times, therefore, do not have a large effect on imaging throughput.

The amplification of rolling circle amplification is extensive so that the signal can be imaged with lower power objectives making acquisition faster (Gyllborg, Langseth, Qian, Choi, et al. 2020; Langseth et al. 2021; Lee et al. 2022; Qian et al. 2020; X. Wang et al. 2018). Furthermore, sub-micron resolution is also not needed since the objects to be imaged are 1 µm in size and that typically 1 cm^2 can be processed. Nevertheless, these methods are not automated so that only one or two rounds are performed manually per day.

For the sequencing-based methods, the spatial throughput should be virtually unlimited. However, these methods have typically small areas of maximum 1 cm^2. In theory the area that can be covered would only be limited by the number of available unique location barcodes. The highest resolution method, Stereo-Seq, uses a 26-nucleotide spatial barcode, which would give a complexity of $4^{26} = 4.5 \times 10^{15}$ unique barcodes. Spreading these out in a square grid with a spot-to-spot distance of 500 nm would give an area of 33.5 x 33.5 meter.

Plenty of space to do spatial transcriptome profiling of three sections of a blue whale. The more realistic limitation to the spatial throughput would be the money required for sequencing the data. Especially since it would scale quadratically when pursuing larger areas.

2.4.3 Sensitivity

For sensitivity, the smFISH methods are leading, and it is thought that smFISH is currently the most sensitive method to count the number of transcripts in cell. smFISH is highly sensitive because of the redundant design, where multiple probes need to bind in order to generate a signal (Raj et al. 2008). If a single probe does not bind the target because of bad design or production, there are many others that will, resulting in little false negatives. Furthermore, if one probe accidentally binds off-target the signal of a single probe is not detectable so that there are little false positives. However, it is very hard to experimentally determine what the actual sensitivity is of smFISH, because it is hard to make a ground-truth measurement. Compared with real-time PCR, smFISH gives similar quantifications (Raj et al. 2008). Likely smFISH is very sensitive to detect RNA molecules that are present in the cell, but the next question then becomes, whether the sample preparation procedure could fix all RNA molecules in place. From cyclic methods we can see that the RNA is not always 100% stable and there is a loss over rounds (Codeluppi et al. 2018), which could also indicate that RNA molecules were lost before the first detection, although hard to prove.

Barcoded smFISH approaches drop slightly in sensitivity to between 84 to 95% of smFISH, due to dropouts and difficulties in recalling the barcode (K. H. Chen et al. 2015; Moffitt, Hao, Bambah-Mukku, et al. 2016; Shah, Lubeck, Zhou, et al. 2016; Xia, Babcock, et al. 2019).

Next are the *in situ* sequencing methods that rely on rolling circle amplification. One downside to enzymatic amplification is the reduction in sensitivity compared to smFISH approaches. Initially there were three enzymatic steps; reverse transcription, ligation and amplification, which each had non-perfect efficiency (Ke et al. 2013; Lee et al. 2014). However, most *in situ* sequencing methods now skip the reverse transcription step and perform rolling circle amplification directly on the RNA which increases the sensitivity somewhat, but it is still below 40% of smFISH measurements (Lee et al. 2022; Sountoulidis et al. 2020; X. Wang et al. 2018).

The array-based sequencing methods have a worse detection sensitivity, and the original Spatial Transcriptomics publication reports a sensitivity of 6.9% of smFISH (Stahl et al. 2016). Since then, the efficiency might have been slightly improved, but remains around or below an estimated 20% depending on the method (Fan et al. 2022; Stickels et al. 2021).

2.4.4 Genomic throughput

For genomic throughput the sequencing approaches are clearly the best. Furthermore, they give the most flexibility because it is not required to pick targets beforehand, as is needed with the imaging approaches. The only initial drawback was that only polyadenylated transcripts could be captured. However, a recent innovation performs *in situ* polyadenylation for non-mRNA molecules so that long noncoding RNAs, micro RNAs, antisense RNAs, small nucleolar RNAs, transfer RNAs and even viral RNAs could be spatially profiled (McKellar et al. 2022).

The first paper that conceptualized barcoded smFISH by the group of Long Cai, proposed that the full transcriptome could be decoded with four fluorophores in eight rounds ($4^8 = 65536$). Practically, this turned out to be impossible due to optical crowding and errors in the barcode. The current highest number of RNA species measured is 10,050 using MERFISH with 69 hybridization cycles (Xia, Fan, et al. 2019), or 10,000 in 80 cycles with seq-FISH+ (Eng et al. 2017, 2019). Nevertheless, typical applications of barcoded smFISH target between a few hundred and a thousand genes. Which is similar to the maximum of 1,022 genes demonstrated for *in situ* sequencing (excluding FISSEQ) (Shi et al. 2022).

Another issue with the targeted imaging methods is that they are slightly restricted by the probe design requirements. Transcripts need to be uniquely identifiable with at least 25 probes with a length of 20 to 30 nucleotides, that additionally, have a similar GC content and melting temperature. This means that for transcripts with many similarities to other transcripts it can be hard to make unique probes, and for short transcripts it can be an issue that not enough probes can be designed. Furthermore, this also means that transcript variants such as isoforms are hard to target. Additionally, some genes are extremely highly expressed with multiple thousands of transcripts per cell, which should be avoided in order to not fill the optical space and jeopardize the barcode identification of other genes.

Thus, the imaging approaches are limited in the number of genes it can cover and to some extent which genes it can target. However, the next question is whether full transcriptome coverage is needed for all questions. For a completely uncharted tissue of an understudied organism, the spatial sequencing approach will be the best solution. However, for tissues where we already have an idea about which genes are active, the number of possible targets is greatly reduced. In fact, most single cell RNA-seq and array based spatial transcriptomic approaches perform a feature selection on the genes for downstream analysis. Typically, only a few thousand variable genes are selected for downstream analysis. In this case the imaging approaches could provide the same information with higher resolution. Nevertheless, it all depends on the question which approach is most suitable.

2.4.5 Adaptability

There is also large variation in the adaptability of methods. Most imaging-based methods require specialized microscopy hardware, and therefore these methods have not spread far from the labs that they were invented in (Moses and Pachter 2022). Furthermore, they usually require dedicated microscopes because the experiments take multiple days, and it is essential that the calibration is maintained so that exactly the same area of the sample can be imaged multiple times. Therefore, it is difficult to run these kinds of experiments on microscopes in a facility, unless they are dedicated to this purpose. Furthermore, the imaging-based methods generate up to multiple terabytes of images per experiment, which requires high power computing infrastructure and image analysis skills to process. These factors make these methods harder to implement.

The array-based sequencing methods do not have the above restrictions and are much easier to implement for other labs. Especially since the protocols just require standard molecular biology steps and DNA sequencing. The latter of which is highly optimized and widely available through sequencing facilities. This also enables the processing of many samples in parallel so that the experimental throughput is high compared to imaging-based methods (Vickovic et al. 2022).

2.4.6 Three dimensions

Most of the methods that are currently out there are applied on thin slices of tissue that are just one cell layer thick, but some imaging methods venture into the third dimension, reaching a thickness of a few hundred micron (Shah, Lubeck, Schwarzkopf, et al. 2016; Wang et al. 2021; X. Wang et al. 2018). Since our tissues are three dimensional, ideally the cells would also be studied in this context. Studying the spatial relationships between two cell types in thin tissue sections, becomes progressively harder the further cells are spaced from each other, because the chance that 2 cells are found in the same thin section decreases radially. A thickness of a few hundred microns using confocal microscopy already gives more power to study these relationships. However, they are challenging to perform due to issues with light scattering at depth and lower resolution of objectives with a longer working distance.

It is however possible to image RNA in three dimensions at cellular resolution in whole mouse brains using optical clearing and imaging by light sheet microscopy, but not at single transcript resolution (Kumar et al. 2021; Tanaka et al. 2020). Furthermore, the imaging time is very long and multiplexing by multiple cycles would render even longer experiment times due to the days it takes for reagents to diffuse in and out of a sample (Murray et al. 2015).

A common approach now to study the spatial transcriptome in 3D is to process multiple sections of a single sample taken periodically along one axis (X. Chen et al. 2022; Ortiz et al. 2020, 2021; Shi et al. 2022). This works both for the imaging and sequencing approaches and enables the study of the transcriptome for anatomical areas. Nevertheless, a caveat is that the 3D environment of a single cell cannot be studied because of the missing sections.

Thus, no good approaches exist yet to locate the RNA in 3D in full organs with single cell resolution. Mostly because of the technical limitations of working with 3D volumes. The sequencing-based methods are inherently two dimensional and their only option to scale to 3D is by sequencing every section. The imaging approaches have the best chance to scale to the third dimension, but they are limited by the tradeoff between working distance and resolution of the microscope objectives. Processing multiple thicker sections with imaging would be an option. However, so far none of the methods has demonstrated the spatial throughput to process such a high volume. As illustration, the largest study processes 40 sections of one hemisphere of the mouse brain (X. Chen et al. 2022), with corresponds to just 2.9% of the total volume. A considerable increase in throughput is therefore needed to enable full murine organ imaging.

All in all, there are a large number of methods out there that all have their advantages and disadvantages, and they should be picked depending on the research question at hand. Furthermore, development will likely continue where methods will hopefully reach cellular resolution, full genome coverage and three-dimensional sampling.

2.5 Applications of spatial methods

Even though method development is ongoing we now find ourselves at an interesting turning point where some published experiments are not just proof-of-principle but are also applied to answer biological questions. Furthermore, the generation of a new type of data consisting of the spatial expression of a large number of genes spurred the development of new bio-informatic tools for exploration and discovery. Here, I will review the application of spatial methods and analysis with a specific focus on the adult and developing nervous system.

2.5.1 Atlasing

Anatomical atlases provide an invaluable reference for scientists because they form the backbone for communicating discoveries. The standardizations of anatomical structures, names and locations give all scientist across the globe a common reference frame into which new discoveries can be placed. Similarly, large scale single cell RNA-seq studies are beginning to provide an *atlas* of cell types that forms a common reference framework for previous and future findings on those cells (Regev et al. 2018; Siletti et al. 2022; Tasic et al. 2016; Zeisel et al. 2018). The development of highly multiplexed spatial methods has the potential to unify anatomical atlases and cell type collections, into one reference atlas as scaffold for that organ or organism (Lein et al. 2017).

The first anatomical atlases of the human brain stem from the early twentieth century and were based on cell densities and morphologies (Brodmann 1909). The *Brodmann* atlas has been updated throughout the years with higher resolution and more detailed annotation in the cortex and brainstem (Amunts et al. 2013; Amunts and Zilles 2015; Ding et al. 2016). For mouse brains the Allen Brain Atlas provides the highest resolution anatomical reference atlas, which is widely used (Wang et al. 2020). Transcriptome information was added by massively parallel application of *in situ* hybridization (ISH) one gene at a time in development (Thompson et al. 2014) and adult mice (Lein et al. 2007). However, human samples are too rare for such an approach, but the highly multiplexed spatial methods provide the solution.

2.5.1.1 Adult brain atlas

As proof of principle, these methods have already been applied on small areas of the mouse brain with imaging of the hippocampus (Shah, Lubeck, Zhou, et al. 2016), somatosensory cortex (Codeluppi et al. 2018), motor cortex (Zhang et al. 2021) and hypothalamus (Moffitt et al. 2018). With the single cell resolution and gene multiplexing of tens to hundreds of genes, the spatial organization of cells in these brain regions could be studied. Most array based spatial sequencing approaches have performed their proof-of-principle experiments on the mouse olfactory bulb as a benchmark, which is therefore extensively profiled (A. Chen et al. 2022; Fan et al. 2022; Fu et al. 2022; Rodriques et al. 2019; Stahl et al. 2016; Stickels et al. 2021; Vickovic et al. 2019, 2022). Depending on the resolution of the method the gene expression could be studied at the level of larger anatomical olfactory bulb structures or at single cell level.

Classical reference atlases typically subdivide the tissue in anatomical regions, for example the different brain areas such as hippocampus, striatum, thalamus and cortex, or even finer details such as the layers of the cortex. These delineations are generated by manual annotation and are based on cell densities or shapes that were acquired by a general cellular stain such as Nissl (Lein et al. 2007). With the highly multiplexed molecular data it is now possible to generate these anatomical annotations in an automatic and unbiassed manner (Codeluppi et al. 2018). A large collection of bioinformatic methods have been developed to generate these spatial annotations robustly and efficiently (Dong and Zhang 2022; Dries et al. 2021; He et al. 2021; Palla et al. 2022; Partel et al. 2020; Pham et al. 2020; Zhao et al. 2021).

With the increase in spatial throughput, it is now possible to process entire sections of the mouse brain (Ortiz et al. 2021), and if this analysis is subsequently repeated for multiple slices, the spatial transcriptome can be reconstructed in three dimensions. For the mouse brain the data can then be aligned to the Allen Brain anatomical atlas so that everyone can benefit from the data (Fürth et al. 2018). The first three-dimensional survey of the mouse brain was done by performing Spatial Transcriptomics on 75 coronal sections of the mouse brain between the frontal cortex and the start of the cerebellum. Based on the spatial transcriptome the data could be regionalized. Furthermore, because of the dense profiling of the tissue volume these regions could be extended to three-dimensional volumes, which had higher detail than the Allen Brain Atlas, demonstrating that the high multiplexing can refine the common reference atlas.

This atlas based on Spatial Transcriptomics, however, has a low resolution of spots of 100 µm. More recently the mouse brain has also been mapped with single cell resolution using *in situ* sequencing (X. Chen et al. 2022; Shi et al. 2022) or multiplexed smFISH (Borm et al. 2022) (part of this thesis). The former performed STARmap PLUS mapping of 1,022 genes in 16 coronal, 3 sagittal and one transversal sections (Shi et al. 2022). The single cell profiles could be clustered, and 231 types were identified that could be related to the clusters found by single cell RNA-seq. The spatial locations of the cells were then used to look for spatial cellular motives, which are neighborhood profiles where a local neighborhood is identified by the cell type composition of that area. These were then clustered into anatomical regions (He et al. 2021), with lower anatomical resolution than the Allen Brain Atlas but it nevertheless provided new insight in anatomical structures in some specific areas.

Another study used another *in situ* sequencing method called BARseq, to measure the spatial expression level of 107 gens in 40 half coronal sections of the forebrain, with a focus on cortical neurons (X. Chen et al. 2022). The segmented cells were then clustered and could be related to single cell RNA-seq studies, as well as aligned to the Allen Brain Atlas coordinate framework. This allowed for the study of cortical types and their distribution. They identified that the largest source of spatial variation can be attributed to variations in cell type compositions in different layers of different cortex areas, rather than variations in gene expression in the same cell types. Furthermore, local gene expression could also be linked to the connectivity of the neurons in question.

A handful of studies applied spatial methods to the human brain, but besides the discussed difficulties with processing human brain due to lipofuscin, no method demonstrated it could scale to full sections of the human brain, which would require an area of roughly 100 cm^2. Therefore, the few studies that mapped gene expression in human brain sections focus only on a relatively small area of the brain, such as a single gyrus of the cortex.

These studies could retrieve the layers from the cortex, either using *in situ* sequencing (Gyllborg et al. 2020; Langseth et al. 2021), MERFISH (Fang et al. 2022) or Spatial Transcriptomics (Maynard et al. 2021). Interestingly, the MERFISH study profiled the gene expression of 4,000 genes in both mouse and human cortex at single cell resolution. They found that cell types and composition were conserved, but cell types had different cell-cell interactions based on soma proximity in the two species. These studies are a start to understand the spatial organization of the human adult brain, but a hundredfold increase in spatial throughput would be needed to profile a single section in full, and therefore the method development should be continued so that we can make an atlas of the human brain and ultimately the entire human body.

2.5.1.2 Developmental brain atlas

A smaller, but not less interesting, sample to study spatially is the developing embryo. Especially since the spatial location of a cell is very important during development because it determines which morphogens and cell-to-cell signaling it receives. The spatial methods are very powerful to begin to generate a comprehensive view of this complex process because it can capture the large amount of patterning factors and organizers in a single experiment.

One study performed single cell RNA-seq of the developing mouse brain between embryonic day 9 up till birth on day 18 and used *in situ* sequencing to locate the organizers that pattern the neural tube in the embryo (la Manno et al. 2021). The experiment profiled 119 genes and was performed on 24 sections of the embryo at day 10.5. This gave a comprehensive overview of complex patterning in the embryonic brain and could spatially locate the cells that form the organizers which were identified by single cell RNA-seq.

In human, a similar approach was used to map clusters of single cell RNA-seq at five weeks post conception to three sections of a human embryo where the expression profiles of 440 genes were measured (Braun et al. 2022). These experiments showed how the neural tube, which mostly consisted of radial glia at this stage, is patterned in the anterior-posterior axis. Furthermore, some radial glia, mostly in the hindbrain, had produced neuroblast and neurons that could be observed in three distinct layers. More focused studies on the developing human cortex have also been performed with either multiplexed FISH (Bhaduri et al. 2021) or ROI based sequencing (Roberts et al. 2021). These studied the development of the layers in more depth and found differences in gene expression between the developing layers but also gradients in the developing cortical sheet which lay down the start of the distinct critical areas.

In mouse there are also three spatial studies that profile full embryos. The first uses DBiT-seq, the microfluid barcoding approach of rows and columns, to look at gene expression and proteins in the embryonic day 10 mouse embryo (Liu et al. 2020). The second used sci-Space that placed an array with spatial barcodes onto a mouse embryo tissue section followed by single nucleus RNA-seq to locate cells in space (Sanjay R. Srivatsan et al. 2021). The last performed Stereo-seq that uses a spatially patterned barcode array of DNA nanoballs to study embryonic development between embryonic day 9.5 and 16.5 (A. Chen et al. 2022). These experiments were more proof-of-principle for these technologies, and they do show automatic data-driven atlasing but not yet at a higher resolution than standard anatomical atlases of these tissues. Nevertheless, they add the dimension of full transcriptome coverage and will hopefully in the future generate highly detailed datasets of development.

3 RESEARCH AIMS

The overarching goal was to develop highly multiplexed RNA detection methods that could be applied in brain tissue sections of mouse and human to map cells and their types in space.

Specific goals for the presented papers:

Paper I

- Optimize smFISH to work on brain tissue samples.
- Develop cyclic smFISH for higher gene multiplexing on brain samples.
- Study cellular organization by mapping cell types in space.

Paper II

- Speed up *in situ* detection of RNA to enable processing of larger areas.
- Build an automated pipeline to perform experiments in a streamlined manner.
- Develop data structures and analysis tools for spatial data.
- Enable smFISH to work on human brain samples.

Preliminary data

- Apply the developed method on human embryo.
- Study patterning of the developing brain.
- Study spatial gene expression in three dimentions.

4 RESULTS & DISCUSSION

4.1 Paper I: Spatial organization of the somatosensory cortex revealed by osmFISH

At the start of this project there was one demonstration of cyclic smFISH on cultured cells (Lubeck and Cai 2012). However, compared to tissue samples, cultured cells have much less background, are easier to permeabilize and label with smFISH. Therefore, we set out to develop a cyclic smFISH protocol that could work in tissue samples. We first optimized existing smFISH protocols that were developed for other tissues, to work reliably in mouse brain sections (Itzkovitz et al. 2011; Lyubimova et al. 2013). The new method is called ouroboros smFISH (osmFISH) and has accelerated hybridization kinetics by making the RNA more accessible by iso-propanol fixation or a heat-shock. Furthermore, we reduced tissue autofluorescence by clearing the tissue without affecting the RNA stability. This enabled the imaging of single RNA transcripts of up to three genes in tissue sections with clear and quantifiable signal.

Next, we implemented a stripping step, using the DNA duplex destabilizing amide, formamide, to melt the fluorescent smFISH probes off their RNA targets, so that all probes can be washed away, and the RNA returns to its original non-fluorescent state. This then allows for another round of smFISH on the same tissue sample. The procedure of RNA labeling – imaging – probe stripping, can then be repeated for a new set of three genes per cycle, until all desired genes are interrogated. To enable cycling we had to ensure tissue stability over all rounds. An often-occurring issue was that the tissue would detach from the sample glass, likely due to swelling and shrinking by osmotic forces, because of different salt concentrations in the buffers. We solved this by maintaining the same salt concentration in almost all steps of the protocol and optimized the sample glass coating so that the tissue would be covalently attached to the surface.

Furthermore, for a cyclic protocol it is very important that the exact same tissue area is imaged in every cycle. We developed a flow cell that can be repeatedly placed in the well-plate adapter of the microscope stage with only minimal displacements between rounds. Additionally, we built a semi-automated fluidic system that could automate parts of the protocol to reduce hands-on time and the chance of human error. In order to image the dim smFISH signal we compiled a microscope system that could detect RNA, clearly separate RNA molecules labeled with different fluorophores and automatically image an area using an automated stage and Z-controller.

We performed the cyclic detection of 33 genes in 13 cycles where we pushed the capabilities of the microscope to image an area of 3.8 mm^2 of the mouse somatosensory cortex. This resulted in roughly 5 terabyte of raw imaging data, for which we build an automated image analysis pipeline that could extract the RNA localization and gene identity from these images, as well as align them between the different cycles and stitch the individual fields-of-view into one image. We also took images of the cell nuclei and soma, which were used to segment the cells and assign the RNA molecules to individual cells. These single cells expression profiles could then be clustered, and the resulting groups could be matched to cell types identified by single cell RNA-seq.

With the cell type identities and spatial location of all cells in the tissue we could build a spatial cell type map. With this map it was possible to study the spatial relationships between cells, where some cell types like excitatory neurons showed very strong self-association, meaning that they are closely located to other cells of the same type in the tissue. In contrast, other types, like microglia and certain inhibitory neurons, show a self-avoiding pattern where they are evenly distributed throughout the studied tissue. Furthermore, we could see that all cell types are always close to endothelial cells of the blood vessels, so that they are close to a source of oxygen and nutrition. Additionally, we could use the spatial locations of the cell types to automatically subdivide the tissue into regions that neatly captured the layered organization of the mouse cortex.

With osmFISH we enabled cyclic smFISH on tissue sections to study the organization of cell types. The linear scaling of the number of targets per cycle gives an easy to interpret approach for multiplexing that is not inhibited by the constraints for low expressed genes for barcoded experiments. However, the gene throughput is limited compared to barcoded methods, to a few tens of genes. Nevertheless, the data is of high quality due to the sensitivity of smFISH and it was possible to perform *de novo* clustering of the single cells and study the spatial organization of the mouse brain.

4.2 Paper II: Scalable *in situ* single-cell profiling by electrophoretic capture of mRNA using EEL FISH

As previously discussed, a large downside of the smFISH approaches, such as osmFISH, is that the signal needs to be imaged with high power objective lenses. Even though this gives very high resolution, the imaging throughput is prohibitively slow. As example, the 3.8 mm^2 imaged of the mouse somatosensory cortex, which is just a fraction of an approximately 70 mm^2 coronal mouse brain section, was imaged in over 13 hours. Moreover, this area needed to be imaged every cycle so that the entire experiment took 13 days to complete. The largest cause for the slow imaging was the requirement for imaging a large Z-stack of around 40 planes for every FOV and color channel.

To solve this issue and enable the imaging of entire mouse brain sections in manageable time, we developed EEL FISH. Taking inspiration from the array based spatial transcriptomic technologies where the RNA is transferred to a capture array, we sought to *blot* the RNA from a tissue section onto a flat substrate, so that the Z-stack could be virtually eliminated. However, unlike the array-based sequencing approaches, where the RNA is randomly diffusing in the hope to be captured by the surface, EEL FISH uses the fact that RNA is negatively charged to actively move the RNA towards the surface by electrophoresis. This additionally also reduces the lateral displacement of the RNA and gives a better cellular signal. After the RNA is captured on the surface, the tissue is digested and encoding probes are hybridized to the RNA encoding a 16-bit barcode which can identify up to 448 genes per color channel.

The removal of the tissue also sped up the detection chemistry because reagents do not need to diffuse through the dense tissue matrix. Together with the reduced Z-stack and improvements on microscope hardware, EEL FISH enables the imaging of one square centimeter in just over two hours. This speed-up enables the processing of a full mouse brain tissue section in just over two days while maintaining the benefit of the high resolution given by high-power objective lenses.

With the expression profiles of 440 genes measured in a sagittal section of the mouse brain we could study regionalization of the brain by subdividing the tissue area in a hexagonal grid and clustering the expression profiles of the tiles. This analysis recapitulated the known anatomical organization of the mouse brain and validated our technology. However, the issue with clustering for regionalization is that this analysis gives discreet clusters which appear equally different from each other when visualizing. This was solved by also showing gradients in regional identity. Furthermore, we also defined a metric to measure the strength of borders in the tissue based on local gene expression. This showed that between some regions there was a very strong border, meaning that the two regions differ highly in gene expression profile, while other regions contained multiple gradients.

By imaging the nuclei before tissue digestion, EEL FISH allows for single cell segmentation. Clustering the single cell profiles clearly showed how most cell types were spatially restricted to certain anatomical locations or structures, while other types were found throughout the brain.

We also developed a fully automated cyclic FISH robot that could independently perform all decoding cycles by automated fluidics and integration with the microscope. This enabled us to perform EEL FISH routinely and we processed seven sagittal sections of the mouse brain, measuring 168 genes to make a mouse brain atlas. Integrating this spatial data with single cell RNA-seq data allowed for the imputation of unmeasured genes. Furthermore, the atlas could be used to transfer annotations from the single cell data to the spatial data and we used this to make a map of where each neurotransmitter is used in the brain.

Lastly, since in EEL FISH the tissue sample is removed after RNA transfer, we also remove most of the highly auto-fluorescent lipofuscin that is found in human brain samples. This allowed EEL FISH to be applied on a gyrus of the human visual cortex measuring 445 genes.

With this information we could cluster RNA molecules with a graph neural network approach to find cell type signatures in space without cell segmentation.

All in all, EEL FISH is a high resolution barcoded multiplexed smFISH method that can be applied on large tissue samples. The speed up and automation enables the routine processing of samples to map the spatial transcriptome and is an important step towards processing human samples. Furthermore, we developed all hardware and software to generate, visualize and analyze large datasets. All protocols, system building instruction and data analysis code are available in high detail for the community to use.

5 PRELIMINARY DATA

We have applied EEL FISH on samples of human development to study patterning and cell type organization in the growing brain. Here I will show the datasets and discuss the analysis.

5.1 Human development 5 week

The first dataset consits of three section of a full embryo with an age of five weeks post conception (Figure 11). This dataset is part of a BiorXiv preprint (Braun et al. 2022) and the presented figures are part of draft figures for a manuscript in preparation by Braun *et al*.

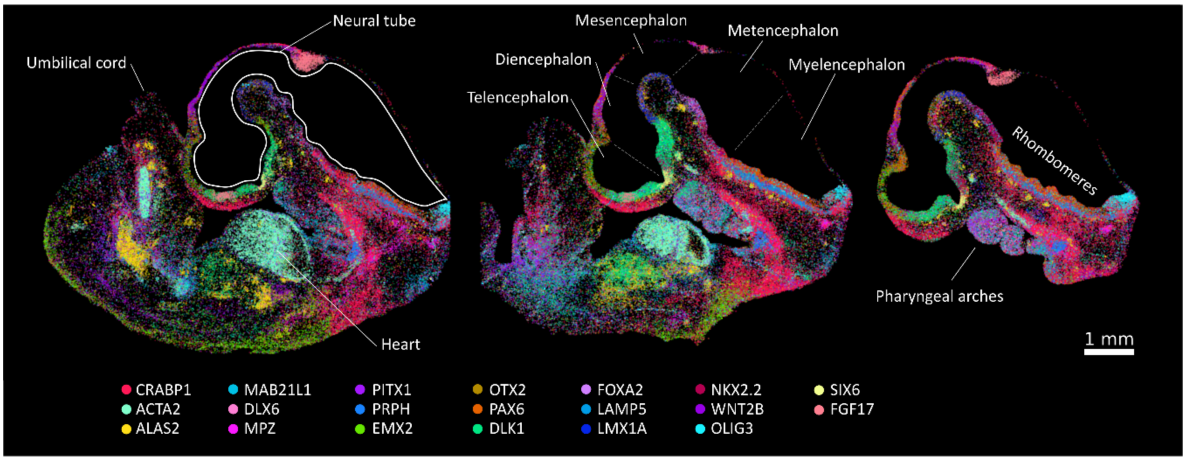
Neural tube
Umbilical cord
Heart
Mesencephalon
Metencephalon
Diencephalon
Myelencephalon
Telencephalon
Rhombomeres
Pharyngeal arches
1 mm
● CRABP1
● ACTA2
● ALAS2
● MAB21L1
● DLX6
● MPZ
● PITX1
● PRPH
● EMX2
● OTX2
● PAX6
● DLK1
● FOXA2
● LAMP5
● LMX1A
● NKX2.2
● WNT2B
● OLIG3
● SIX6
● FGF17

Figure 11 | Three EEL FISH datasets of the human embryo at five weeks post conception. Sections are cut in the sagittal orientation and various anatomical structures are indicated. Each dot is a single molecule of RNA and the color corresponds to one out of 20 selected genes out of 440 measured genes.

At five weeks post conception the basic building plan of the embryo has been layed down. At this stage the future brain consits of a closed neural tube, that has a clear bend in the future midbrain (mesencephalon). Also other regions of the prosencephalon are anatomically identifiable by neural tube thickness and bends between regions. In the hindbrain (metencephalon and myelencephalon) the rhombomeres can be recognized by their periodic wavy pattern. Most cells in the tube at this stage are radial glia wich form a layer of cells closest to the venticle. However, some neuroblast are present and are mostly found in the hinbrain at this stage. In the ventral myelencephalon for instance, a clear layer of neuroblast excists marked by LAMP5 (blue), which are positioned ventrally of the radial glia marked by PAX6 (orange) in this area.

By morphology the major brain segments are clearly visible. However, by looking at the gene expression of the radial glia along the neuroepithelium, it is aparent the the patterning is even more detailed. Figure 12 shows the gene expression of the radial glia starting posteriorly on the dorsal side, and moving anteriorly where it loops around the forebrain to move posterior again on the ventral side. The plot is subdivided by the prosomeric model based on morphology and gene expression. The anatomical colors corespond to the Allen brain developmental mouse atlas.

By the diagonal it is clear that each region has one or multiple genes specifically enriched in each segment. These genes clearly follow the anatomical annotation and are highly specific. Furtheremore, some areas such as the pallium and diencephalon could possibly be further divided based on their gene expression. For instance the posterior part of the pallium speems to be marked by the RSPO2 and RSPO3 genes. Conversely, there are also genes that mark multiple regions and there are genes that are expressed in both the ventral and dorsal side of the same prosomere. Especially in the istmus and midbrain there seems to be a high degree of ventral - dorsal symmety. For example, WNT1, EN2, PAX5 and PAX8 are expressed on both sides in this section. However, there are also a few more specific genes such a FGF17 that is expressed only in the dorsal side of the midbrain.

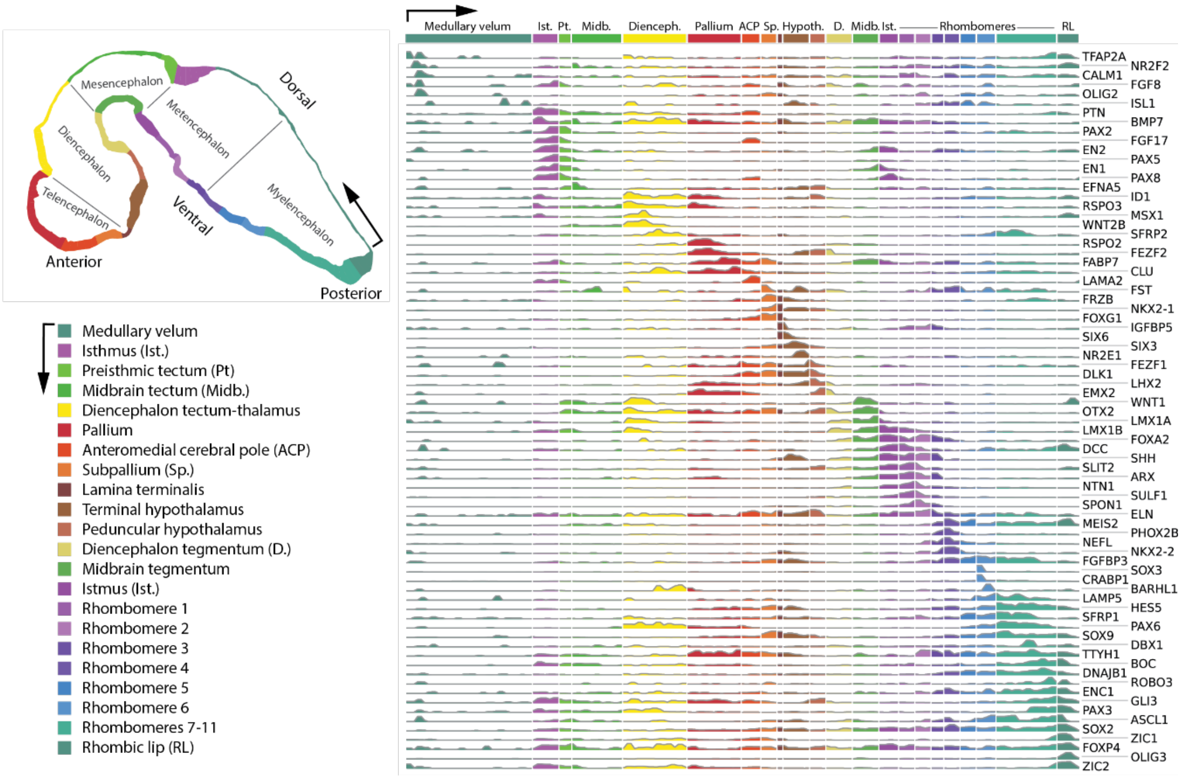

Figure 12 | Patterning in the neural tube of the human embryo at five weeks post conception. On the left the anatomical annotation according to the prosomeric model is indicated. On the right the gene expression along the neural tube is plotted. The line follows the neuroepithelium along the neural tube in the direction indicated by the arow.

5.2 Human development 7 week

The second dataset comprises 24 sections of the full head of a human embryo at seven weeks post conception. The 24 sections were sampled sagittally of one half of the head between the midline and the most lateral point. The inter-section interval is approximately 150 µm. This dense sampling gives a complete view of the developing head and allows for 3D interpretation.

Between the five- and seven-week datasets there is a huge anatomical difference due to the rapid development of the embryo. Figure 13 shows the 24 sections where the head is outlined in blue and the brain in orange. Three genes are shown to indicate the brain (DCX and EMX2) and the connective tissue of the head (DCN).

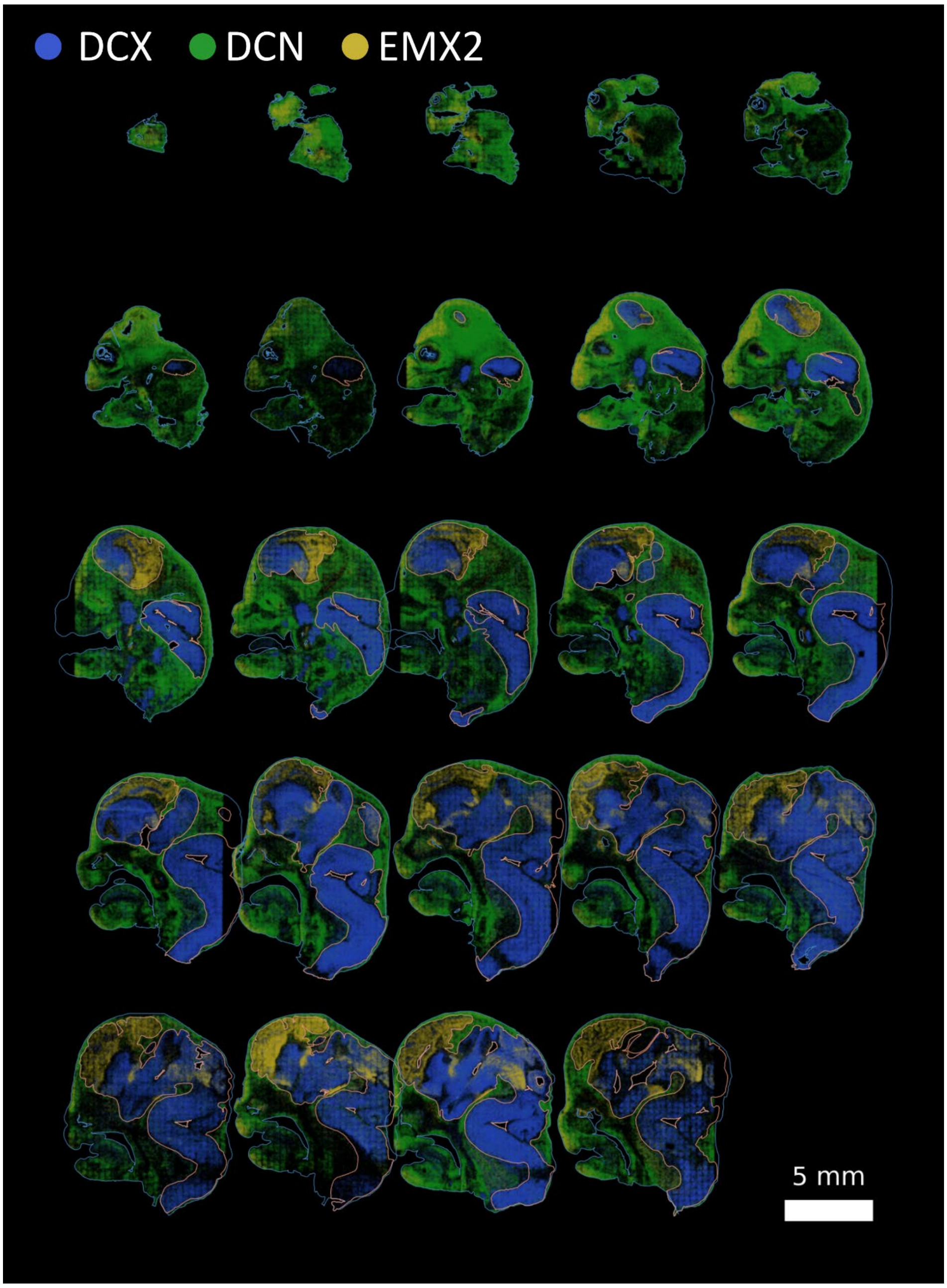

Figure 13 | 24 sections of the developing human head at seven weeks post conception. The sections are arranged from the most lateral section in the top left, to the most medial section in the bottom right. The nose is oriented to the left. Raw gene expression is shown where DCX labels most of the brain and EMX2 labels predominantly the developing cortex. DCN is expressed outside of the nervous tissue.

In figure 14, the radial glia in the ventricular zones are shown by SOX2 and PAX6 expression. These areas are still relatively thin, similar to the five-week sample. However, their progeny has increased substantially, for instance the neurons labeled by DCX and EMX2 cover large areas (Figure 13) and are responsible for the large anatomical difference seen between the two datasets.

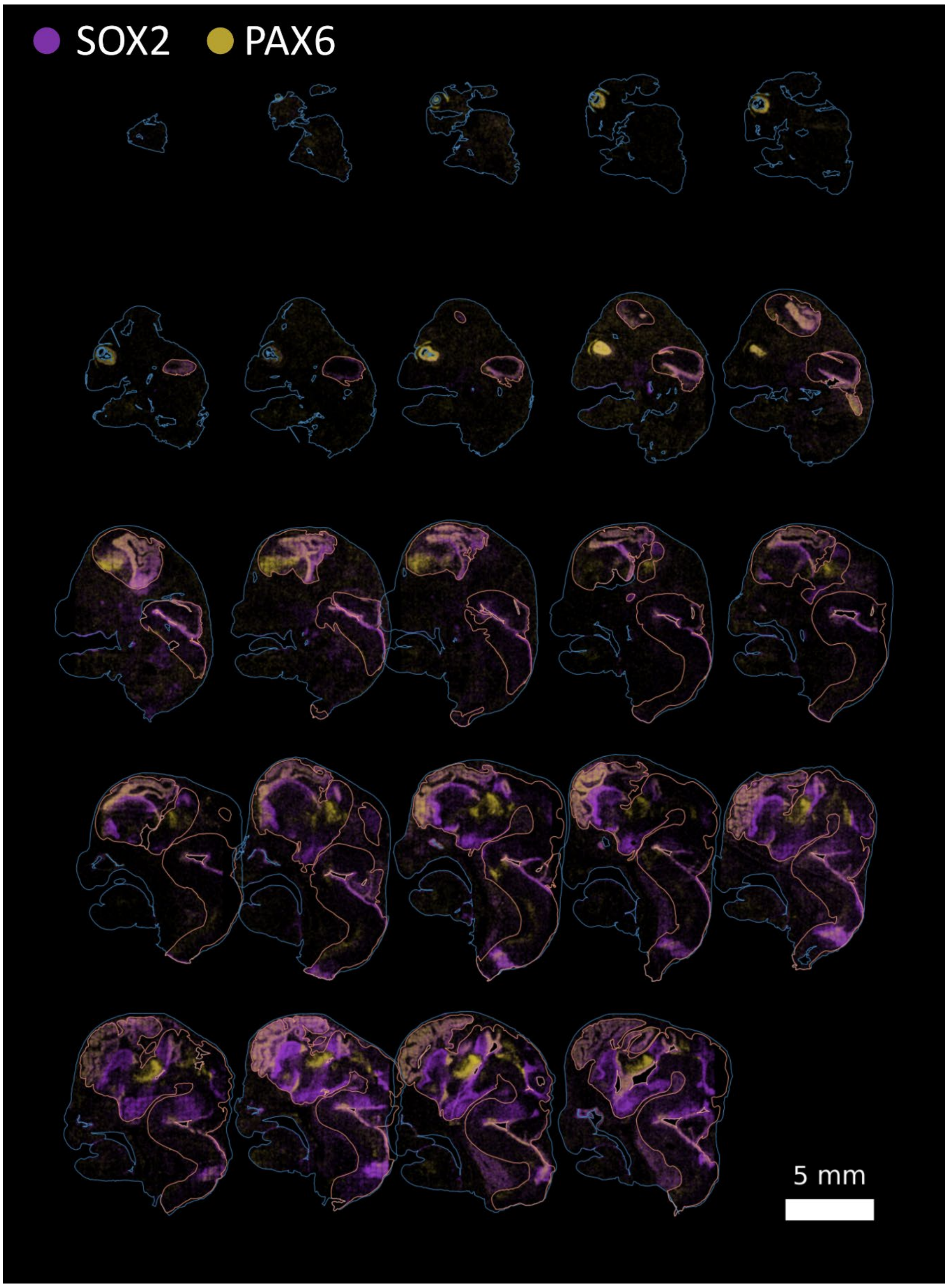

Figure 14 | SOX2 and PAX6 label the radial glia located in the ventricular zones of the developing brain.

As a result of the detailed patterning seen in the five-week dataset, we see a similar complex picture in the seven-week dataset (Figure 15). The patterned areas have expanded and gave rise to distinct daughter cells. One goal of this project is to identify each region in the developing brain and link it to existing knowledge of developmental trajectories.

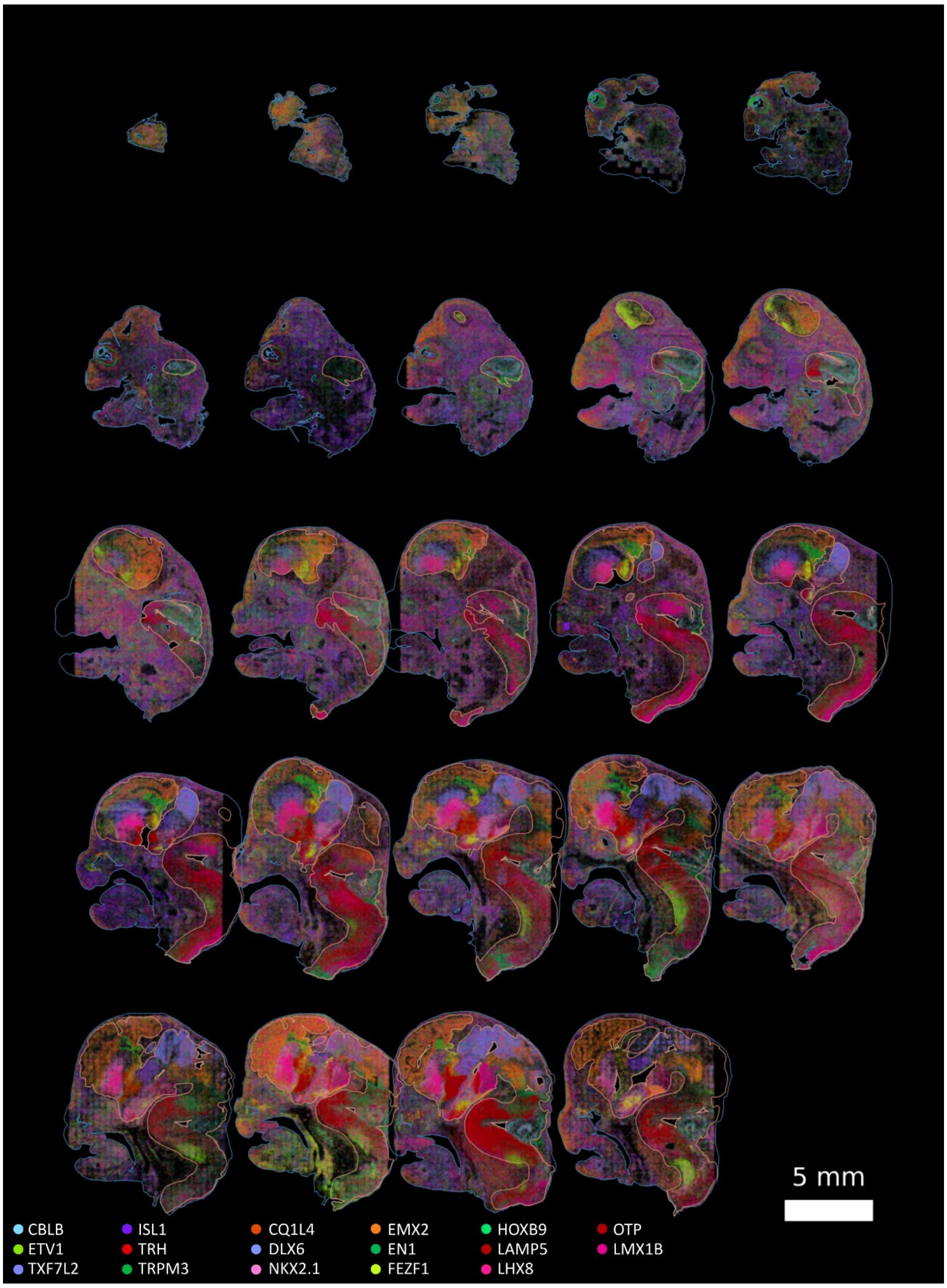
5 mm
● CBLB
● ETV1
● TXF7L2
● ISL1
● TRH
● TRPM3
● CQ1L4
● DLX6
● NKX2.1
● EMX2
● EN1
● FEZF1
● HOXB9
● LAMP5
● LHX8
● OTP
● LMX1B

Figure 15 | Expression patterns of 20 selected genes that are expressed in highly specific areas of the developmental brain.

One way to study the patterning is to regionalize the dataset. To do this the RNA localizations were binned in a square grid of 70 µm. The resulting tiles were subsequently clustered to group pixels with similar expression profiles together. This resulted in a data driven anatomical annotation of the head (Figure 16).

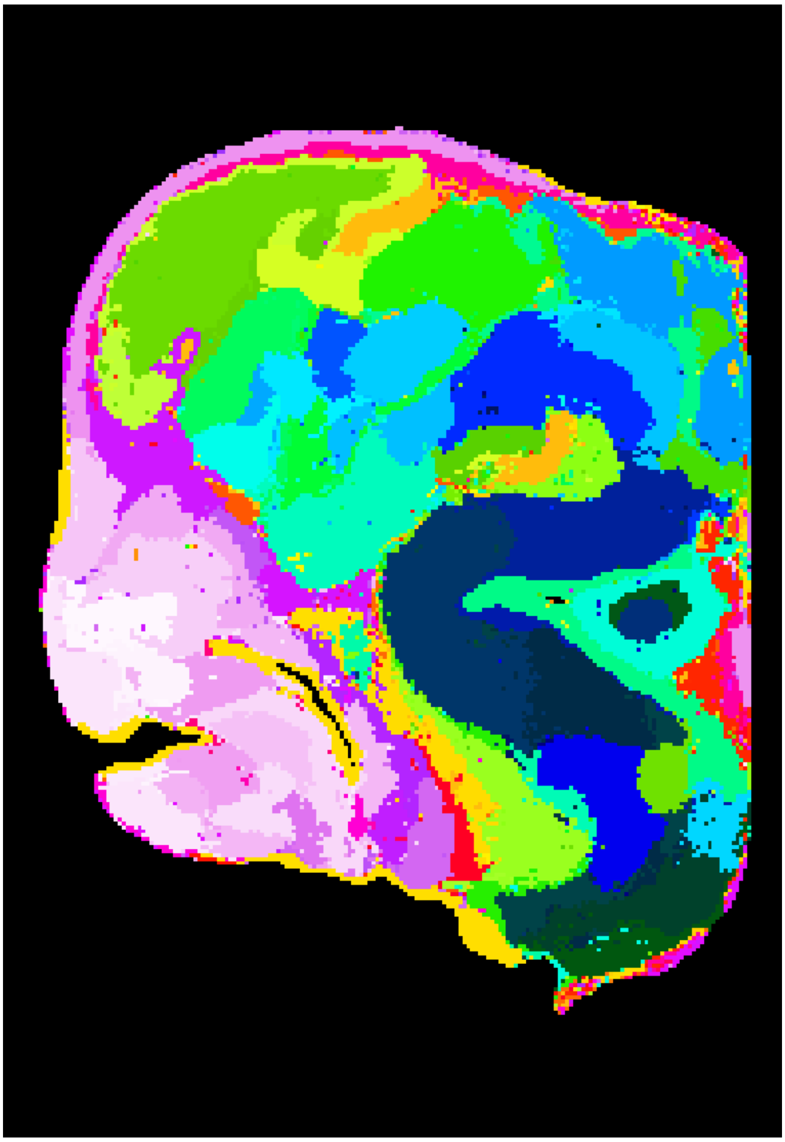

Figure 16 | Regionalization of one section of the dataset where each color corresponds to a single cluster or region. Color similarity indicates similarity in expression profile.

However, since the dataset contains 24 sections I wanted to perform the regionalization in 3D. The first step was to align the different sections that due to cryosectioning artifacts had warped. To do this, a mean morphology was calculated between adjacent sections using an image warping algorithm (Figure 17). The image was created by merging the expression profiles of a number of high contrast genes. Calculating the likely morphology of the original slice happened in two steps. First the four adjacent sections on the left and right were morphed to generate two mean images on either side. These were subsequently used to make a second mean image which should be what the slice in question should have looked like assuming that the averaging corrects for random distortions due to the cyrosectioning process. Then the warping parameters were calculated that would warp the raw data into the mean morphology, and this was used to warp the gene expression data for all 440 genes. This process was then repeated for each slice in the dataset and resulted in a better alignment in 3D.

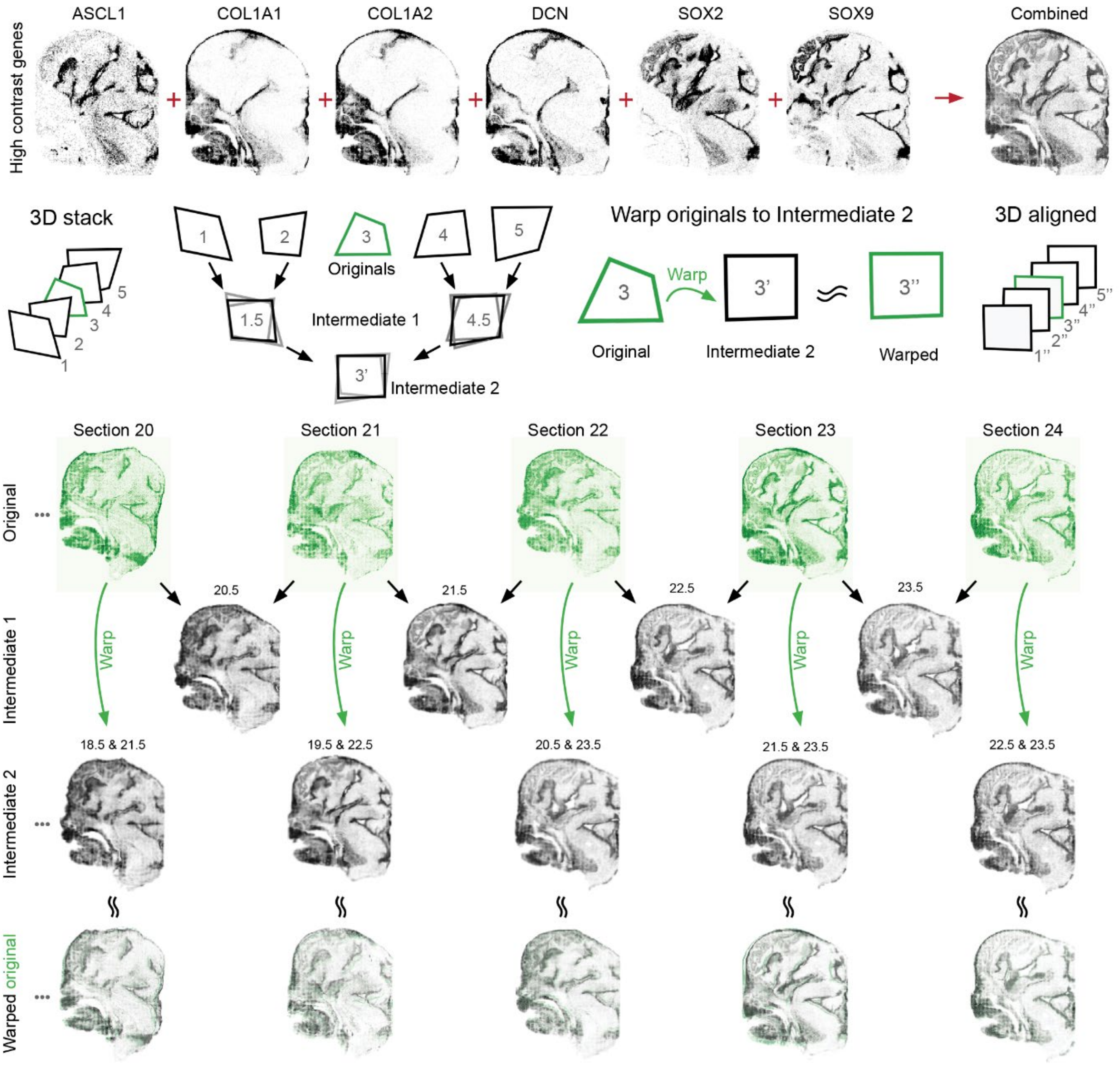
ASCL1
COL1A1
COL1A2
DCN
SOX2
SOX9
Combined
High contrast genes
3D stack
1
2
3
4
5
Originals
1.5
4.5
Intermediate 1
3'
Intermediate 2
Warp originals to Intermediate 2
3
Warp
3'
3''
Original
Intermediate 2
Warped
3D aligned
1''
2''
3''
4''
5''
Section 20
Section 21
Section 22
Section 23
Section 24
Original
Intermediate 1
20.5
21.5
22.5
23.5
Warp
Intermediate 2
18.5 & 21.5
19.5 & 22.5
20.5 & 23.5
21.5 & 23.5
22.5 & 23.5
Warped original

Figure 17 | Schematic representation of algorithm to warp individual tissue sections so that they better align in 3D. First, genes with high contrast are taken and combined to generate an image that can be used for image warping. The images of the unaligned sections are then taken to calculate two layers of intermediates. The second intermediate is the product of the adjacent four images. The data is then warped to the intermediate two. This is done for each section so that they align to each other in 3D.

During sectioning of the sample, the distances between slices were recorded and this could be used to place all sections in 3D space using a regular grid of 50×50×50 µm voxels. Since the average inter-section distance is 150 µm there are some missing slices, which was corrected by interpolating the gene expression data in 3D. This resulted in a full 3D gene expression map for 440 genes, containing 74 slices of which 24 were actually measured.

This data could then be clustered to render a 3D data driven anatomical map of the developing head (Figure 18). Annotation of this dataset will provide a complete view of the complex patterning in the embryonic head. Where for each region the gene expression profile is known. This shows the power of highly multiplexed smFISH to enable the study of complex processes and generate interpretable data.

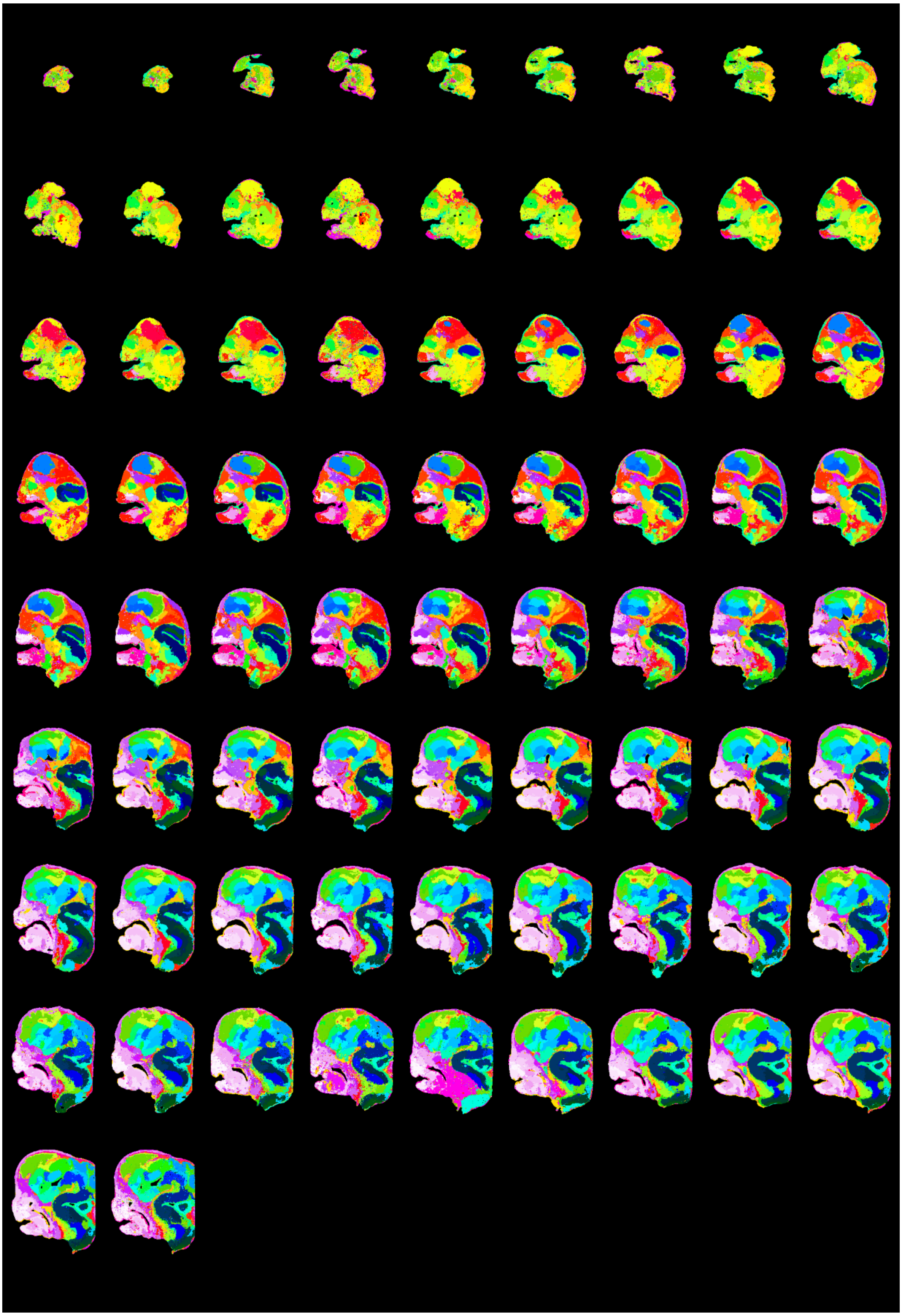

Figure 18 | 3D regionalized map of the developing head showing the measured and interpolated data aligned in 3D.

This work is coverd by ethical permits 2020-02074, 2020-03045, 2019-04595 and 2018/769-31

6 CONCLUSION & PERSPECTIVES

The work presented in this thesis is part of the development of the young but quickly growing field of spatially resolved transcriptomics. In the early phase there were proof-of-principle demonstrations of cyclic smFISH but these were conducted on cultured cells, where the spatial context is much less interesting than in tissues. The first part of the thesis therefore focused on developing a method for cyclic smFISH that could be applied on tissue sections. By optimizing the sample processing and developing an experimental setup we were the first to break the color barrier by cyclic smFISH in tissue samples (la Manno et al. 2016). We then implemented automation and extended the multiplexing capability to make the osmFISH method presented in **Paper I**. Applying this method on the mouse brain generated high quality gene expression profiles that could be used to make a cell type map and study spatial organization of a complex mixture of cells. Additionally, this dataset is still used as benchmark for other detection and bioinformatic methods.

However, we realized that the imaging bottleneck in smFISH approaches would prohibit these methods to scale past a few mm^2, so that the applications for these methods would be highly limited. Therefore, we sought to improve the spatial throughput by reducing the amount of imaging needed, without making concessions in resolution so that barcoding of a large number of genes would be minimally affected by optical crowding. The EEL FISH method presented in **Paper II** enables the study of full mouse brain sections at single cell resolution to study spatial gene expression, cell type organization, regions and borders of the tissue. With robust automation, EEL FISH enables the routine study of large tissue sections of 1 cm^2 where the expression patterns of 448 genes can be measured per used fluorophore. The transfer of the RNA onto a surface substantially reduces the required imaging in the Z-dimension and speeds up the detection chemistry, while not sacrificing resolution to maximize optical space for barcoding. This combination of speed and resolution enables high multiplexing that could scale to thousands of genes and allows for imaging of entire mouse organs. Currently EEL FISH has simultaneously demonstrated one of the largest covered areas with the highest spatial resolution of all published spatial methods. The only drawback is that the detection efficiency is lower than for in-tissue smFISH, but still on-par with spatial sequencing methods. Additionally, the fact that EEL FISH enables the study of human adult samples without the need for additional experimental procedures is an important step towards making a human cell atlas that will serve as a valuable reference atlas to accelerate discovery in human samples.

However, building such an atlas would require an additional scale up of the method. If we would start with the human brain. A single section of roughly 100 cm^2 would be 100 times larger than what we currently process with EEL FISH. With the current data acquisition speed, it would take 8.8 days to image the section once. A full 16 cycle experiment would thus take approximately 142 days, or 4.4 months, to complete. It is unlikely that with the current protocol, the sample is stable enough to last for that long, although it would be interesting to try. Furthermore, the data generated would pose another substantial problem. I estimate that this experiment would generate roughly 300 terabytes of raw image data. This would be challenging to process but not impossible if highly parallelized computing architecture were used.

Thus, an additional speed-up of roughly two orders of magnitude would be needed to enable the processing of full sections of the human brain or other organs for that matter. Considering that from osmFISH to EEL FISH we already implemented a speed up roughly two orders of magnitude it is conceivable that with a few years of work, processing 100 cm^2 would become feasible.

The bottleneck would still be the imaging and the data acquisition speed could be reduced in a number of ways. As mentioned, with the latest generation of cameras the largest part of the imaging time is spent on mechanical movement of the XY stage, Z drive, auto-focus and filter turret. Enabling faster integration of these elements could speed up the imaging. Furthermore, in our current EEL FISH protocol we still need to take a small Z-stack due to the fact that the flow-cell is bending the sample glass. By designing a better flow-cell the Z-stack could be reduced to a single plane. This would also enable stage-scanning, where the stage moves at a constant speed and images are made continuously to reduce mechanical movement time. With these improvements I believe EEL FISH could already scale one order of magnitude to 10 cm^2 in the same experimental time. Further development will be needed to speed up the imaging even more.

Other technical challenges for the field would be to implement 3D mapping of cells and their transcriptome. This challenge, however, is highly limited by the capabilities of microscopy to image signal deep into a tissue. The most promising approach would be light-sheet microscopy, but there is currently no method that can combine high resolution imaging at depth. Possibly a hybrid method, where the sample is sectioned in a few thicker slabs depending on the imaging depth, could be imaged at high resolution and later stitched back together.

Another interesting challenge is to combine multiple modalities in the same experiment to do a multi-omics measurement. There are already a number of examples where proteins, chromatin conformation and histone modifications are detected alongside transcriptome measurements (Codeluppi et al. 2018; Deng et al. 2022; Mateo et al. 2019). This could be further extended to measure the state of the cell more completely.

Now that the imaging-based methods are scaling their gene throughput to many thousands of genes and the array-based approaches are improving their spatial resolution to sub-cellular level. Spatial methods could soon replace single cell RNA-seq because they generate equivalent information but with the added benefit of the spatial dimension. Furthermore, the imaging-based methods will also scale better and more cost effective when more cells need to be processed compared to sequencing. As example for the human embryo dataset at 7 weeks post conception, a single experiment contains typically 500,000 cells which are processed in just 3 days of imaging, with the cost per cell one order of magnitude cheaper than single cell RNA-seq. However, the gene throughput is much lower, but increasing the number of genes would not increase the cost of the experiment substantially. Therefore, the spatial methods that have only recently been developed are already very competitive with single cell RNA-seq and could soon become the favorite method to profile individual cells.

The spatial dimension will be valuable for understanding biology, considering the fact that tissue architecture is so tightly regulated and crucial for organisms to function properly. Already now the available data can be used to learn how a diverse set of cell types is spatially organized to form distinct regions or how cell types are related to each other. More information about how tissues are built, relative occurrences of cell type numbers and knowledge about cell-cell interactions will give us insight into how the sum of the parts adds up to the total tissue function.

As example of one such finding, the hepatocytes in the liver are spatially organized in a gradient between portal veins and central vein where their position determines which step of the bile acid biosynthesis they perform (Halpern et al. 2017). Cells along this axis express different enzymes for the various production steps and therefore form an organized production line made of cells. Additionally, knowing how cells are organized in healthy samples will also give us insight into posible defects in disease. Cell migration defects and their effects are a good example of this, but also in other diseases there is a spatial component. For instance, in brain tumors an outstanding question is the extent of immune cell penetration of the tumor and why they are quickly inhibited in their function when they do infiltrate. As a second example, in Alzheimer's disease a special activated state of microglia was described that preferentially localized with amyloid plaques (Keren-Shaul et al. 2017).

Furthermore, detailed information about cellular organization in tissues will also be invaluable to enable reconstruction tissues, for instance for the *in vitro* generation of organs meant for transplantation. Similarly, it could also help to fine-tune organoid models to better mimic the actual organ. Organoids grown from induced pluripotent stem cells from patents will likely play a key role in the transition to personalized medicine, and their clinical relevance highly depends on the accuracy by which they simulate the patient.

These goals would also require detailed information about embryonic development which is needed for the *in vitro* generation of organoids and organs. As we have seen, development is a highly complex system where many processes are acting in parallel to ensure that the embryo develops properly. Without the ability to measure all these simultaneously in the same sample it is hard to get a comprehensive view of the ongoing dynamics. The current knowledge is painstakingly compiled from many different experiments studying one process at a time but connecting these is hard because they are studied in different samples. With the development of the highly multiplexed spatial methods, we can simultaneously study how various patterning factors, organizers, cell-cell interactions and chemotaxis generate the organism. As a start of this analysis, the **preliminary data** demonstrates how the high multiplexing sheds light on multiple simultaneous specification processes in the embryo and can clearly reveal the complex patterning occurring in the embryo. Further, analysis is needed but the generated datasets will hopefully be a resource for comprehensive development research that will illuminate the rules of development. Understanding of these processes will help us comprehend developmental defects that could potentially be cured, help us build *in vitro* organ models and in a more distant future hopefully allow us to synthetically design groups of cells to work together for a common goal.

Now that the spatial methods are increasing spatial throughput it will soon also be possible to process entire bodies of smaller organisms, or entire embryos of larger animals such as the mouse. With the current EEL FISH throughput, it would already be possible to process roughly 400 *C. elegans* bodies or a single *D. melanogaster* body by imaging all sections to get an understanding of the location and identity of all cells in the organism in a single experiment. This, furthermore, enables the possibility for screenings of developmental perturbations where the effect can be studied in the full body where cell type locations, quantities and spatial interactions can all be accounted for.

With further speed increases it would hopefully soon also be possible to process entire mice. This would be important because the current biological field is highly fragmented where individual research groups are studying a single organ or the effect of a single gene in a single tissue. These focused efforts are of course important for our understanding, but since all organs of our body are cooperatively linked, knock-on effects of one system to the next could be easily missed if not focused on.

The interconnectedness is especially clear in development where one mutation can cause multiple defects throughout the body and exemplifies the need to studying the entire embryo (Tanteles and Suri 2007). This multi-system effect is either because the mutated gene has a fundamental cellular function so that many cells throughout the body are affected, or because the same gene is used in different context, possibly with different effects in different organs (Paaby and Rockman 2013; Wurst, Auerbach, and Joyner 1994). Furthermore, diseases can also affect multiple systems. Take for instance the debate about the role of the gastrointestinal tract in Parkinson’s disease, of which some argue is the origin of the pathology observed in the brain (Scheperjans, Derkinderen, and Borghammer 2018). Much Parkinson’s research has been focused on the brain and the effects of or on the gut might have been neglected in studying mutations, evaluating models or therapies. Being able to routinely study the full organism will enable studying of the entire effect of mutations and diseases. Especially, if the right computational analysis tools are developed that can quickly identify deviations in cell frequencies and locations.

Therefore, the development of highly multiplexed spatially resolved detection methods, such as presented in this thesis, can provide a holistic insight into biology and will hopefully give rise to the routine full body single cell profiling of organisms. Combined with the generation of reference anatomical atlases using spatial methods, these techniques will hopefully build a solid scaffold to embed research findings of individual cell types, combinations of cells or entire organs into the same framework.

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