SPATIAL ANALYSIS OF COMPLEX BIOLOGICAL TISSUES FROM SINGLE CELL GENE EXPRESSION DATA

Clustering and visualizing functionnal tissues in *P. dumerillii*

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SPATIAL ANALYSIS OF COMPLEX BIOLOGICAL TISSUES FROM SINGLE CELL GENE EXPRESSION DATA

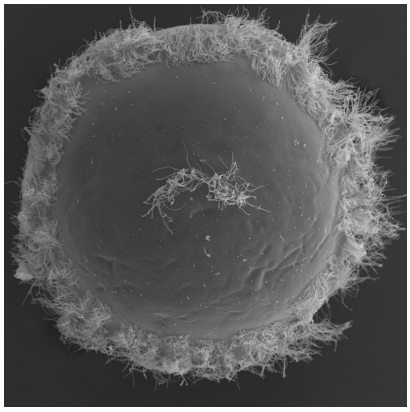
1 CAPTURING GENE EXPRESSION IN *PLATYNEREIS DUMERILLII*'S BRAIN

1.1 PLATYNEREIS DUMERILII, AN IDEAL ORGANISM OF BRAIN DEVELOPMENT STUDIES

1.1.1 *General description*

P. dumerillii is a marine annelid of the class Polychaeta, it has been established as one of the main marine animal models in the fields of evolutionary, developmental and neurobiological biology as well as ecology and toxicology [14, 30, 12, 4, 7, 8]. As a member of the bilateria *P. dumerillii* has a defined bilateral symmetry.

P. dumerilliipopulates shallow (no more than 3m) hard ocean floors around the world. It is commonly found in the Mediterranean sea, the north Atlantic coast of Europe as well as in the shallow seas surrounding Sri Lanka, Java and the Philippines. Eggs, embryos and larvae are roughly 160 µm while the adults can measure up to 6cm in length.



(a) Larval form of *P. dumerillii*. Image: MPI for Developmental Biology.

(b) Adult *P. dumerillii*. Image: Arendt group, EMBL

Figure 1: *Platynereis dumerillii*'s larva and adult forms.

They are several reasons why *P. dumerillii* has been chosen as a model by numerous laboratories. In terms of evolution *P. dumerillii* shows several interesting characteristics. It belongs to the lophotrochozoan taxon of the bilaterian animals as opposed to most of the well established models animal which either belong to the ecdysozoans (*Caenorhabditis elegans*, *Drosophila melanogaster*) or the deuterostomes (mouse, human). Lophotrochozoans being extremely under represented, *P. dumerillii* as a model organism is essential to comparative approach

on bilaterian biology.

P. dumerillii also shows an exceptionally slow evolutionary lineage. It has even been described as a "living fossil" for that reason [8]. This means that the ancestral developmental characteristics of *P. dumerillii* are at an image of the common past of all bilaterians. To illustrate this fact an interesting example described in [3, 31] is the conserved molecular topography of the genes responsible for the development of the central nervous system between *P. dumerillii* and all vertebrates. This slow evolutionary rate confers *P. dumerillii* the advantage of being a link between fast evolving models like *drosophila* and vertebrates.

In terms of practicality, *P. dumerillii* can easily be kept and bred in captivity producing offspring throughout the year [7]. The behavioural characteristics of *P. dumerillii* mating ritual have been well studied. The "nuptial dance" happens on the water surface, male and female releasing the sperm and eggs synchronously, respectively. This activity is synchronized by pheromones released into the water [34]. Over 2000 individuals can be produced within a single batch. Every new individual will undergo embryonic then larval development before reaching *P. dumerillii*'s adult form.

1.1.2 *Larval development*

Similarly to the other polychaetes, the larval development of *P. dumerillii* can be decomposed into three main anatomical stages: the trochophore, the metotrochophore and the nectochaete. The trochophore is spherical and moves via a equatorial belt of ciliated cells as well as an apical organ possessing a ciliary tuft [26, 22] as seen on figure 1a and schematically on figure 2. the metotrochophore stage is characterized by the development of a slightly elongated segmented trunk compared to that of the trochophore [11]. The next stage is the nectochaete larvae that resembles the adult (figure 1b) in most of the traits especially with parapodial appendages used for swimming and crawling [11]. This traditional subdivision has been applied to *P. dumerillii* [13].

Aside from this purely anatomical subdivision, an additional staging systems exists and has become the norm for current studies. The development is measured in *hours post fertilization* (hpf) at 18°C.

A key factor making *P. dumerillii* such an interesting model to work with is the fact that after fertilization, the $\approx$ 2000 larva will start developing at the exact same time, in a synchronous fashion. Furthermore, the larval development of *P. dumerillii* follows a very stereotypical pattern with very little variation from one individual to the other

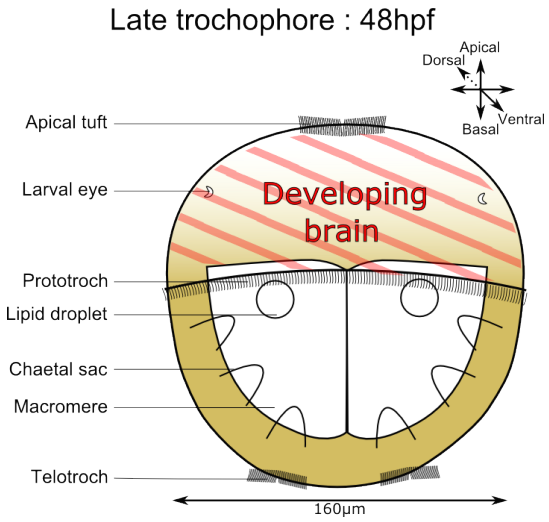


Figure 2: *Platynereis dumerillii*'s larvea development at 48hpf or late trochophore. Striped in red is indicated the area which forms the developing brain of the larvae.

and even between batches provided the temperature is kept constant [7, 4]. An example showing the similarity between individuals during development can be seen on figure 3. this is a very important feature as it allows biologists to repeat experiments on several individuals at a very close developmental stage even if they are from different batches.

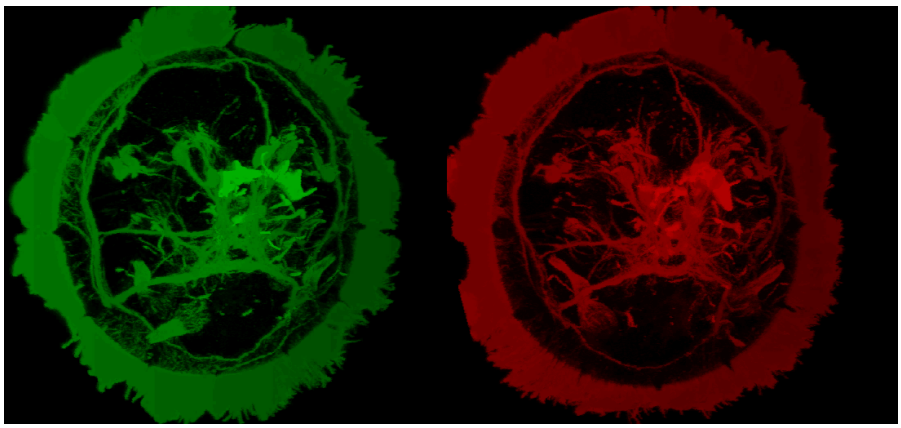


Figure 3: *Platynereis dumerillii*'s stereotypical and synchronous development. In green and red are two different *P. dumerillii* individuals' with the same gene expression being highlighted. They show extremely similar patterns of development.

Describing the entire development of *P. dumerillii* does not fall within the scope of this thesis. Indeed, we will only be interested in the brain of *P. dumerillii*'s larvae at 48hpf. Therefore, it is important to have an anatomical idea of what the brain looks like at this time

in development and what inherent characteristics will be the most interesting to investigate.

1.2 GENE EXPRESSION IN PLATYNEREIS' DEVELOPING BRAIN

1.2.1 *Platynereis' nervous development until 48hpf*

The main purpose of this thesis is not to fully understand the patterns of development in *P. dumerillii*'s larval brain. Therefore we will only give a brief summary of what the main component of the brain are at 48hpf, the time point we will be interested in in the next chapters. *P. dumerillii*'s larval brain development is detailed in [8].
From the early trochophore (24-26hpf) neural system development starts taking place. The apical ganglion forms at the apical tuft. It contains one sertonergic cell and a few neurons that link to the nerve of the ciliary band of the larva called the prototroch (see figure 2 for better understanding). This allows the first movements of the larve thanks to the cialited cells of the prototroch.
The mid-trochophore (26-40 hpf) sees the formation of the first cerebral commissure, it is a band of nerves interconnecting the ventral nerve cord and the brain. This trait is a typical feature of annelids brains. During this phase the apical ganglion becomes bigger with three more serotonergic cells.
The late trochophore (40-48hpf)sees the formation of the second commissure in the ventral nerve cord. It is at the end of this stage that the brain starts to become more complexity with a notable increase in the number of neutrites.
The data we will use in the rest of this thesis will not encapsulated the whole larvae, just the brain (see figure 2) thus excluding the ventral part of the nervous system. The best studied areas of the brain are the larval eyes, the developing adult eyes, the apical organ on the dorsal side. On the ventral side are located the mushroom bodies a pair of structures that are known to play a role in olfactory learning and memory in insects and annelids [32]. A schematic representation of those areas is shown on figure FIGURE brain areas.

Even at a very early stage in a relatively simple organism, the brain quickly becomes a complex tissue. Cell types diverge and functional areas are formed. Before trying to understand more about *P. dumerillii*'s brain organization, it is interesting to ask the more general question about how complex tissues such as the brain are defined spatially.

1.2.2 *Spatial organization of complex biological tissues like the brain*

This section is not intended to demonstrate a specificity of the *P. dumerillii*'s brain, it is meant to ask some of the fundamental questions that intrinsically motivate the work presented in the rest of this thesis. Complex tissues, the obvious example of which is the brain, could be viewed as an interconnected mosaic of cells having different functions, working together to achieve the global function of the organ.

If we look closely at this mosaic of cells, the spatial organisation of this mosaic is not random. Cells that serve the same function will often be close from each other, thus defining functional tissues. However, the spatial coherency of those tissues is not necessarily always the same. Some cell types could be formed of cells scattered inside another more spatially coherent tissue. To illustrate that fact, an interesting example is the difference between the spatial coherency of cells forming the neuronal tissue in the brain and cells forming a well defined region in the brain like the mushroom bodies. When asking the question, is it likely that this cell is fully surrounded by the same cell type, the extensions created by the axons of neurones will decrease this probability. Indeed, axons will grow through other types of tissues to reach their destination, making the overall spatial coherency of "neuronal" tissue smaller than very well spatially defined tissues.

When trying to analyse the full structure of the brain with an automated method, keeping in mind that fact could prove important to improve the results. This fact and its consequences on the work presented in this method are further discussed in section cite section spatial clustering.

So far, we have only regarded organs and cell types as regarding their anatomical traits. But as mentioned in the introduction **??** the functional heterogeneity of complex tissues goes further that simple anatomical traits. We need to work on traits that fundamentally represent how cells are functioning.

1.2.3 *Generalities about gene expression and development*

When speaking about developmental biology it it should be noted that the term “cell" will be referring to eukaryotic cells and more specifically those of multicellular organisms. Every cell in a complex organism possesses the same genome, that is, the sum of all the genetic information contained in the cell (nucleus and other compartments). This fundamental homogeneity is in plain contradiction with the heterogeneity observed anatomically. If every cell has the exact

same DNA, where does the great variability between cell types come from (what makes a neurone become a neurone and not a pancreatic cell). Answering this sort of questions defines the field of developmental biology.

The short and rather complete answer to any developmental biology question actually is: same genome but different pattern of gene expression. As indeed gene expression is the central, most important, most studied cellular activity. Gene expression even is general common denominator of life as large parts of the mechanisms making up gene expression are actually shared by every living creature known to man.

Of course to understand what gene expression is, we must first define what genes are. The precise definition of a gene is still controversial. The concept of a "factor that conveys traits from parents to offspring" was laid by Gregor Mendel in 1866 [18] when the accepted theory at the time was based on blending inheritance where the traits of the parents appeared mixed in the offspring following a continuous gradient. The most recent published definition of a gene followed the publication of the ENCODE project [6]. It states that a gene is "A gene is a union of genomic sequences encoding a coherent set of potentially overlapping functional products."

Gene expression is the way cells express their genes. Expression of a gene is the process of transcribing the DNA of that particular gene. The product of gene expression is RNA molecules and there are several ways to look at gene expression. In a cell or tissue, at a given time point we can to choose to look whether a gene is expressed or not (binary expression) or how much a certain gene is expressed (quantitative expression).

Most RNA molecules are translated into proteins that can have very different purposes some will directly serve in the cellular life as functional/structural agents (elements of the ATP synthase for example) others will have a regulatory effect on gene expression. In other terms the expression gene a, coding for protein A might activate, accelerate, inactivate or decelerate expression of gene b and potentially others. This outlines the complex interdependent regulatory system that is gene expression. For precise examples gene regulation see [10, 27, 9, 2].

Add figure for gene expression Camille

During development mechanisms exist that allow gene expression to become differential as the divisions occur. This is how the asymmetrical axis (dorso-ventral, and basal-apical) of the body are defined.

The main mechanism involves chemical gradients. The first of these gradient has to come from the original cell which musty contain some asymmetrically distributed chemical so that the first divisions lead to non identical cells. In the case of *Platynereis dumerillii*, the body axis are defined between 2hpf and 7hpf [8].

As described, gene expression is the key factor during tissue development. The ability to study gene expression patterns has revolutionized the fields of developmental biology. Technological innovation has been the main driving factor of this revolution. In the next section we will present two methods to capture gene expression.

1.3 CAPTURING GENE EXPRESSION IN THE LABORATORY

1.3.1 *In-situ hybridization assays*

In-situ hybridization (ISH) is an experimental technique where the practitioner is able to determine in which cells of the tissue under study a particular RNA is expressed. As opposed to Southern blotting, ISH assays not only allow to know whether a gene is expressed or not, but also where in the tissue it is expressed. First proposed in 1969 by Pardue [23] and John [15] independently, in-situ hybridization (ISH) used radioactive tritium labelled probes on a photographic emulsion to reveal on which chromosome particular genomic components were located. With the development of fluorescent labelling techniques [16, 24] allowing for faster, more sensitive and of course safer hybridization assays [28] compared to radioactive probes, Fluorescent in-situ hybridization (FiSH) quickly became the standard technique to study gene expression in the spatial context of the biological tissue. Importantly, using multiple fluorescent probes of different colours allowed the simultaneous localization of several RNA fragments in the tissues [21].

1.3.2 *Building a image library of gene expression for Platynereis*

During his PhD, Raju Tomer and colleagues cite thesis member of the Detlev Arendt lab in EMBL, used Fluorescent in-situ hybridization to create an image library of gene expression in the brain of *P. dumerillii*. He was able to record gene expression in the full brain at 48hpf for 169 genes. In practice each individual larvae was dissected to isolate the brain, which was then cut into thin slices and fixed. Each individual slice was then stained with two different fluorescent probes corresponding to two messenger RNAs (RNAm). One of the gene is considered a reference, as it is always hybridized in all the assays (the main reference gene used was Emx) along an other gene of interest,

see figure 4.

As mentioned previously, the larval development of *P. dumerillii* is highly similar in every individual larvae. In the case of this study requiring a lot of different assays conducted each time a on different animal, the stereotypical development of *P. dumerillii* has proven essential. Indeed, having the same reference localized in all the assays has allowed Tomer to align all the other gene expression patterns onto this scaffold.

The result is an image library of 169 gene expression pattern in the full brain of *P. dumerillii* with a exploitable spatial reference that allows for a very precise mapping.

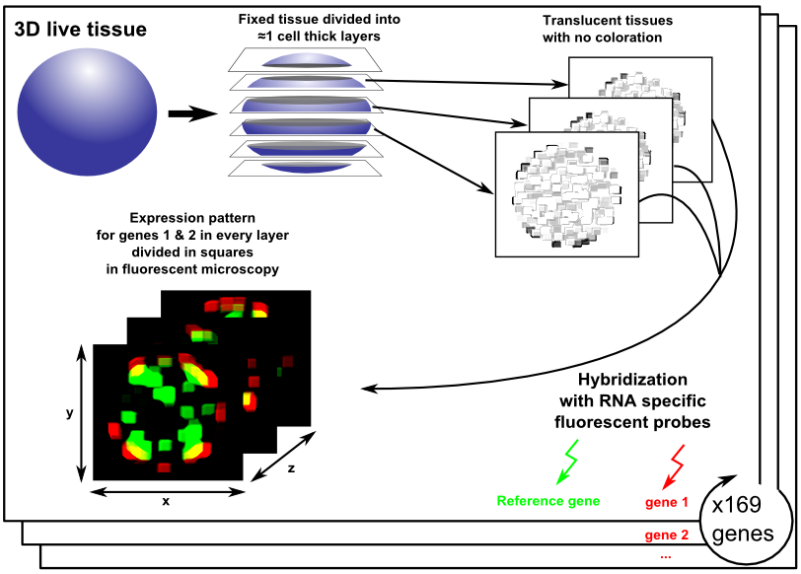


Figure 4: Fluorescent in-situ hybridization assays to create a 169 genes catalogue of gene expression in the brain of *P. dumerillii*. From the live tissue cut into thin fixed layers, every slice is stained with a reference gene and a gene of interest that will reveal the areas of expression under fluorescent microscopy. The process repeated 169 times for key genes in *P. dumerillii* development has been generated by [32]

However useful and practical fluorescent in-situ hybridization may be, such assays are limited in terms the quantity of gene one is able to study. Indeed, each individual larvae only provides the expression of two genes, one being the reference. Crucial developments in sequencing technologies have brought a way to study the expression of the whole transciptome landscape in a single assay, RNA sequencing.

1.3.3 *RNA sequencing*

Whole Transcriptome Shotgun Sequencing (WTSS) also called RNA sequencing (RNA-seq) [19, 33] has developed alongside Next Generation Sequencing (NGS) technique used to retrieve the genome (DNA). Instead, when preparing the starting material, only the RNAs are extracted. If interested in protein coding messenger RNA, they are separated from the rest by targeting the polyadenylated 3' tail, specific to protein coding transcripts. Most current technique use magnetic beads to achieve this separation [20, 19].

Once isolated from a population of cells, transcripts undergo fragmentation to obtain an average length of 200-300. The next step is then reverse transcription, which will create a complementary DNA (cDNA) library using a viral reverse transcriptase enzymes. After amplification using quantitative Polymerase Chain Reaction (qPCR), the cDNA library is ready to be sequenced by NGS technology.

This will generate a large dataset of small reads, that need to be mapped back onto the reference genome of the considered species, providing this genome is available. In that case the resulting dataset will reflect a snapshot of the whole transciptome in the studied cell population. However, in the case of *P. dumerillii*, this reference genome is not fully available yet, an alternative option being to map the reads back to a list of known gene sequences, for instance the 169 genes studied by [32] (PrimR genes). The obtain dataset will represent a quantitative image of the considered genes in the cell population at one point in time.

Because of technical limitations in this sequencing protocol, until very recently the starting quantity of RNA had to be relatively important. This is why most of the published RNA sequencing studies use a population of cell as a starting point. This however, means that the gene expression landscape obtained as an output will represent an averaged expression over all the cells used as an input.

Importantly, when comparing RNA-seq the the previously described in-situ hybridization technique, if the methodological burden to analyse the expression of a lot of genes at the same time is greatly reduced, the spatial localisation of the cells is lost during the protocol.

1.4 CONCLUSIONS

In this chapter we have presented *Platynereis dumerillii* and the advantageous traits it exhibits for developmental biologists especially in the

field of neural development. We have discussed the fact that anatomical traits are not sufficient to fully comprehend the deep heterogeneous patterns of functionality inside a complex organ such as the brain. In order go push this understanding further we need to take an interest in what defines the life of tissues and sub-tissues, gene expression. We have also described two methods that allow practitioners to capture gene expression from a biological tissue, and how an image library of gene expression for 169 gene was generated by [32] in the full brain of *P. dumerillii*.

So far, our scale of study has been the tissue, or the sub-tissue. However, as mentioned in the introduction **??**, the heterogeneity of complex biological tissues does not stop at this scale of study. In fact, with a top-down approach looking at big tissue and then separating them in smaller sub-tissues until "true" functional tissues are defined is an extremely complicated approach. A solution to this problem would be to actually reverse the approach from a top-down to a bottom-up mindset. This mean reducing the scale of study to the smallest biological unit we can work with, the single cell, define the heterogeneity of gene expression at the single cell level and work our way up to the functional tissue level. Instead of a fragmentation problem, we would have a clustering problem, attaching single cells to a certain number of categories. In order to implement such an approach, what we need is single cell gene expression data.

2

FROM TISSUE TO SINGLE CELL TRANSCRIPTOMICS, A PARADIGM SHIFT

2.1 SPATIALLY REFERENCED SINGLE CELL-LIKE IN-SITU HYBRIDIZATION DATA

Dividing images into "cells"

Because in-situ hybridization keeps the studied tissue spatially untouched, achieving single cell gene expression resolution from one image obtained through fluorescent microscopy is a matter of microscope performance and cell size. For big enough cells, single cell resolution has been documented as far as 1989 [29] with some work specifically directed towards achieving this single cell resolution [25].

When considering [32] dataset, with current microscope technology, achieving single cell level resolution in *P. dumerillii*'s brain on one particular image is feasible. However, our main limitation is the quantity of data involved, indeed, each brain is separated into 20 slices, for 169 genes. This technical bottleneck can be overcome with an automated way of analysing the fluorescence images. However this is not an easy task, as the computer program required needs to be able to "see" and divide the global picture into cells. Considering that all cells do not exhibit the same shape and size, constructing this "cell model" is a very complicated task.

Possibilities exit to highlight the limits of the cells and to automatically acquire those boundaries through computer vision. They rely on targeting proteins in the membrane or in the extracellular matrix of the cells with specific fluorescent probes. Once the boundaries are acquired, defining every cell is a matter of finding enclosed spaces. To that end, numerous contour detection algorithms exist [17, 5, 1].

Unfortunately, a dataset with the cells limits highlighted does not yet exist for *P. dumerillii*'s brain, making a precise division of the images into cells very difficult. Instead, Tomer used a basic approach to divided the images, the "cube" model [32].

A simple cell model, the "cube" data

Every slice of *P. dumerillii*'s brain being aligned onto the reference gene scaffold (see section 1.3) for all 169 genes, the "cube" model

simply consists in dividing each image into square approximately the size of an average cell. In our dataset, the size chosen was 3 μm^2. Importantly, this is actually smaller than the average cell size in *P. dumerillii*'s brain. each slice of the brain being approximately 3 μm thick, the resulting dataset, referenced on a 3-dimensional axis, will contain 3 μm^3 cubes, each of those attached to the luminescence data for 169 genes. - FIG 4 : From images to luminiscence cube data - Present the cube cell model, and its assumptions - cells have roughly the same size - cells are roughly cubical - Present the choice of size for the cubes (3um or 6 um) - This model introduces errors (cells divided or several cells in one cube, empty cubes between cells) - => When working on this data we will need methods that are able to smooth those mistakes over.

2.2 SINGE CELL RNA SEQUENCING, BUILDING A MAP OF THE FULL TRANSCRIPTOME

Sequencing single cell RNA contents

- Same as tissue sequencing but with a lot less starting material - Present the main techniques used (see with Luis) : Microfluidics and others - We obtain the full transcriptome of every cell sequenced

Mapping back gene expression to a spatial reference

- Single cell RNA-seq at the moment does not allow to track cell localization - Need to map the transcriptome back to a spatial reference - Use in-situ hybridization results as reference

2.3 ABOUT THE QUANTITATIVE TRAIT OF SINGLE CELL EXPRESSION DATA

Light contamination in in-situ hybridization data

- FIG 5 : show light intensity across one slice - Explain problem of scale and light contamination

Technical noise in single cell RNA-seq data

- FIG 6 : show "typical" correlation plot from single cell RNA-seq with the noise increasing when reducing starting material - Both methods are currently unreliable quantitatively => need to binarize

2.4 BINARIZING GENE EXPRESSION DATASETS

Binarizing in-situ hybrdization datasets

- With biological knowledge and a limited number of genes - Possibility to compare spatially the resulting binary expression patterns to microscope data and adjust for each gene the threshold manually

Binarizing whole transcriptomes

- Manual curation no longer possible - Thresholding ideally with density peaks - Problems that may occur and possible solutions (figure?)

2.5 PRELIMINARY RESULTS ON MAPPING SINGLE CELL RNA-SEQ DATA IN FROM PLATYNEREIS' BRAIN

Single cell RNA-seq in Platynereis' brain

- Present the data (number of cell) - Present the method used (to dissolve the brain, to capture the cells, to sequence the cells

Mapping back RNA-seq data back to PrimR in-situ hybridization assays

- Select the overlapping genes - Present mapping method (Nuno's pipeline) - Present simple mapping technique and why it is not satisfactory - Present John's method - FIG 6: find a nice way to show a few good examples of mapping

Part II

APPENDIX

APPENDIX A

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