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Neuro-evo-devo in the single cell sequencing era

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Abstract

The nervous system represents the most complex tissue in animals. How this complexity evolved has been a challenging question to address. The explosion in single cell sequencing techniques, the development of new algorithms to cluster single cells into cell types, along with powerful tools for drawing developmental trajectories offer a unique opportunity to compare homologous cell types between species. They further permit the identification of key developmental points and transcription factors that can lead to the evolution of new cell types. At the same time, the ease of use and efficiency of CRISPR genome editing technology allow validation of predicted regulators. This promises exciting developments in the next few years in the field of neuronal evolution and development.

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Introduction

Single cell sequencing gives access to transcriptomic information for every cell in any animal at nearly every developmental stage. Over the last five years, a number of computational algorithms have been generated to cluster single cell transcriptomes into different cell types in an unbiased manner [1–4], to match cell types between different species [5], and to generate developmental trajectories for these cell types [6–13]. The development of such tools offers us a unique opportunity to address the molecular developmental mechanisms that lead to the evolution of new cell types. In this review, we suggest how newly developed computational tools applied to single cell transcriptomic data can be used to study cell type evolution in neuronal systems.

How do cell types evolve?

Cell types were historically defined as a population of cells sharing the same morphology, and, when known, biochemical properties and function. In the transcriptomic era, similar transcriptomes are another feature that cells should satisfy in order to be classified as the same cell type. The inherent noise of transcription, the effect of cell cycle, as well as the response to potential stimuli can cause the transcriptome to be significantly more diverse than the morphology; two cells identical in terms of morphology and function will never have identical transcriptomes, which makes their classification into cell types more challenging.

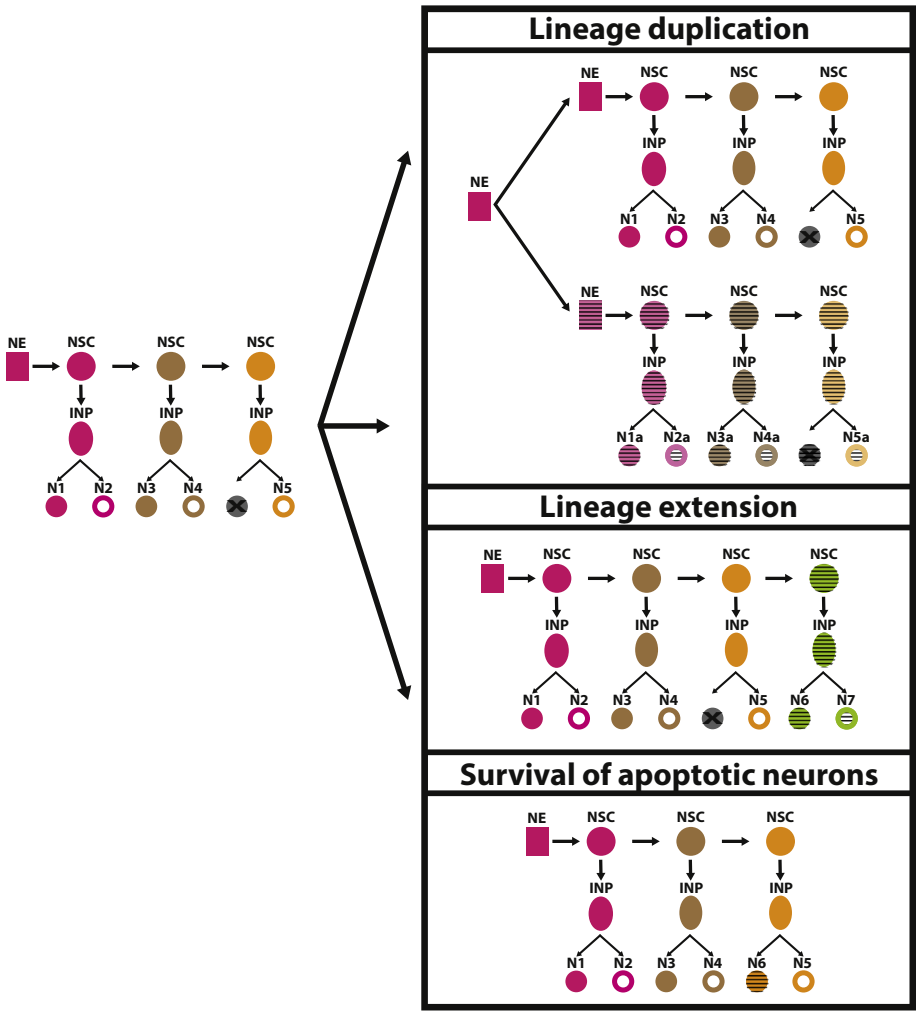
Today, large consortia have been initiated to identify all the main cell types in human, mouse, and fly tissues thus defining 'cell atlases' [14]. These efforts will generate the knowledge needed to understand the extent of variability within cell types and determine whether some cell types that are thought to be distinct may actually represent elements of a larger continuum [15]. Thus, cell type classification may be seen as a taxonomy of the cells in an organism.

Whether cell types are distinct or form a continuum, it is important to understand how they evolved to give rise to the differences that we observe today (Figure 1). Lineage duplications or extensions can generate supernumerary cells of the same cell type that can then diverge from one another and sub-functionalize [16]. Moreover, apoptosis is a normal component of developmental processes in which cells that are not needed are purged during development [17]; their survival could underpin the evolution of new cell types [18] (Figure 1). Other ways could be envisaged for the evolution of new cell types.

Neuro: single cell mRNA sequencing of neuronal cell types

The nervous system consists of a large number of different cell types that are tightly interconnected to form functional circuits. Understanding how this amazing diversity has evolved under the constraints imposed by connectivity is still defying our technical abilities. The newly developed massively parallel single cell mRNA sequencing (scRNA-seq) techniques have allowed researchers to read the transcriptomes of all cell types in neuronal tissues. Three main approaches are used to sequence single neurons: a)

Figure 1



Evolution of development of neuronal cell types. Different events may lead to the evolution of new neuronal cell types. Duplication of a lineage doubles the amount of neurons that are generated from it; the duplicated neurons can then diversify from the ancestral ones and acquire new functions. The same is true in the case of lineage extensions, where the production of more neurons generates potential for cell speciation. Another alternative is the survival of apoptotic neurons that can then acquire distinct identities. New cell types are indicated by the horizontal stripes.

droplet-based techniques (Drop-seq [1] and 10xGenomics [19]) allow for the parallel sequencing of a large number of single cells at a relatively low depth, b) plate-based methods and Fluidigm [20] are used to sequence many fewer cells at increased sequencing depth per cell, and c) split-pool combinatorial barcoding [21] can be used to increase the number of single cells that can be sequenced in a single experiment. Moreover, single-nucleus techniques have also been beneficial for sequencing neuronal types in vertebrates, avoiding lengthy digestions with proteases for tissues that are complex and hard to dissociate [22]. After acquiring the transcriptome of each cell, clustering algorithms, such as density clustering, k-nearest neighbor algorithms, etc, allow the grouping of single cells into clusters of transcriptionally similar units. Finally, cluster annotation (correspondence of each cluster with a cell type) has to be performed *a posteriori* and relies on identifying markers that separate the clusters from each other and annotating the cell types that express these markers. A good previous knowledge of the tissue and its morphologically and functionally-defined cell types is thus very beneficial. Using these transcriptomic techniques, many different neuronal tissues have been sequenced at the single cell level over the last two years, greatly increasing our understanding of cell type diversity and development [2,3,22–33].

Evo: how to assign homologous cell types using single cell sequencing

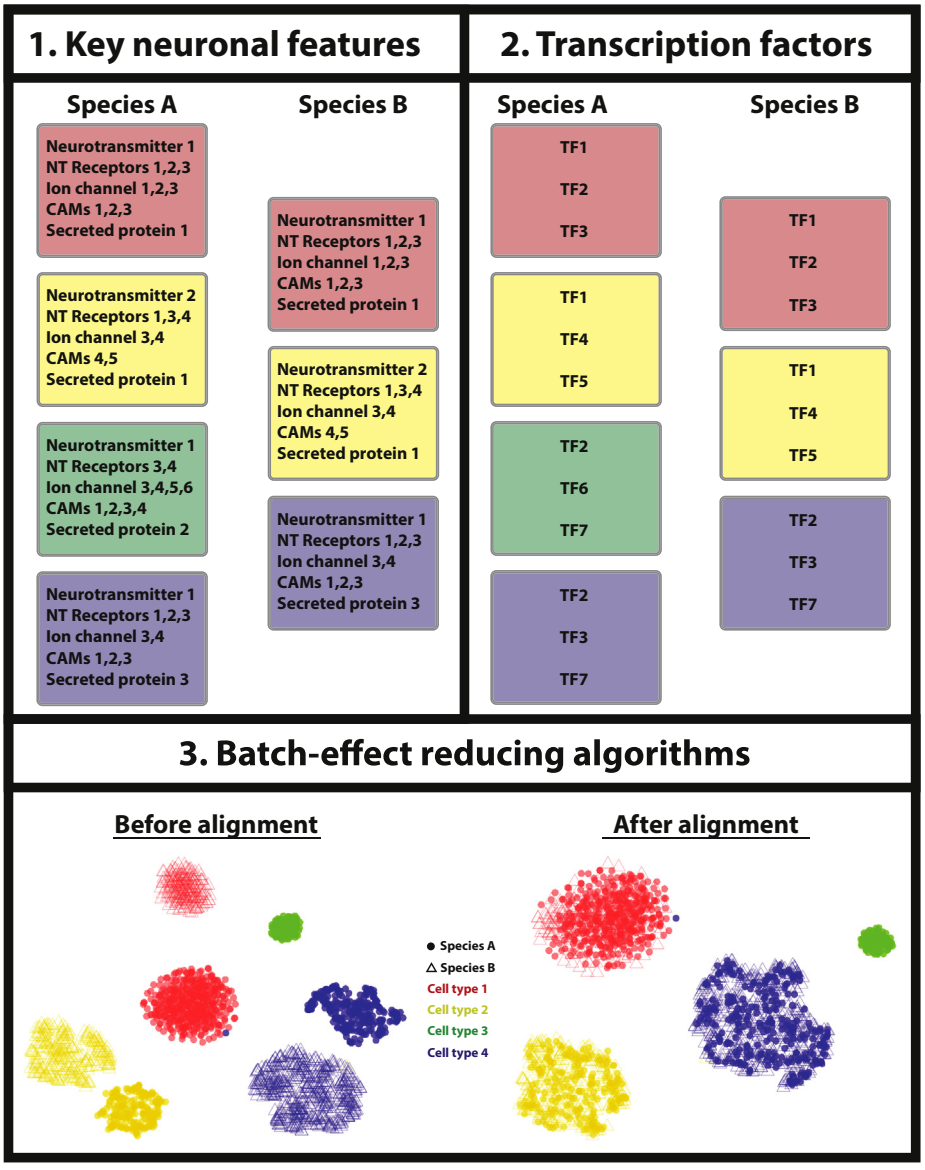
One of the major difficulties in studying cell type evolution is to identify homologous cell types between different animals. A major issue in comparing

transcriptomes between animals is assigning orthologous genes [34,35] and normalizing their expression across species [36]. A number of different approaches have been proposed to homologize cell types from one animal to another (Figure 2):

Identification of key features that endow each neuronal type with its functional characters

One approach to compare neuronal cell types between different species is to uncover a collective set of effector genes that are directly responsible for the specific neuronal features of each cell type and to compare their expression across species. Such features could be neurotransmitter identity, neurotransmitter receptors, ion channels, cell adhesion molecules, etc (Figure 2). This necessarily relies on identifying orthologous genes, which can be a difficult task in the case of large gene families, such as neurotransmitter receptors that often have many different subunits. To overcome this problem, scmap [37] uses an unsupervised approach to select

Figure 2



Identification of homologous cell types. Three different approaches can be used to match homologous cell types between different species: 1) one can use key features that define each neuronal type, *e.g.* the expression of specific neurotransmitter, neurotransmitter receptors, ion channels, cell adhesion molecules (CAMs), secreted proteins etc. If the different species' cell types express the same functional molecules, they are most likely homologous. 2) Alternatively, one can select a core regulatory complex of transcription factors and compare their expression between cell types of different species. Homologous cell types should express the same transcription factor fingerprint to regulate their phenotypic characteristics. 3) Finally, one could apply batch effect correction algorithms before comparing the different cell types between the two species, with the assumption that the effect of their independent evolution resembles a technical batch effect.

features that are representative of the underlying biological differences [38], which are then used to project data sets from one experiment onto a reference data set of another. Such approach was shown to reliably match pancreatic cell clusters coming from different single cell sequencing studies [37]. It remains to be shown whether scmap can match cell clusters from distinct species.

Identification of a core regulatory complex of transcription factors that defines a cell type

Transcription factors control the morphology, physiology, molecular characteristics, and hence the identity of a neuron. Therefore, the unique combination of transcription factors can be used to define cell types more precisely than the combination of effector genes that are often shared by multiple cell types. One way to compare cell types between different species would be to define a cell type-specific transcription factor identity (core regulatory complex or CoRC [16]) and use this for assigning homologous cell types (Figure 2).

This could be done either in a supervised or unsupervised manner. If the tissue of interest has been studied extensively, one could select transcription factors that are known to specify different neuronal features, such as the case of terminal selectors in *Caenorhabditis elegans* [39]. Alternatively, one could use unsupervised models to detect combinations of transcription factor present in single cell clusters and then select those that appear to define a cluster in different species.

Use of batch-effect reducing algorithms

Experimental results are affected by technical sources of variation, known as "batch-effect", which may compromise data analysis. One could consider the differences in expression between the same cell type in different species as the batch effect of an experiment performed by evolution. In this case, an approach to compare cell types from different species would be to apply batch effect correction algorithms before aligning the two datasets (Figure 2).

A number of batch-effect correction methods have recently been developed [5,40,41]; however, only one such algorithm has been used to integrate data from different species [5]. It relies on identifying a shared gene correlation structure that is conserved between the data sets. It can then spot cells that cannot be well described by this shared structure, which can be used to identify cell types that are non-overlapping between the data sets. It then aligns the data sets into a conserved low-dimensional space, using nonlinear 'warping' algorithms to normalize for differences in feature scale, in which one can perform comparative analysis to identify changes in population density or gene expression. This is an active area of research and, as new data from different species are being generated, new algorithms will emerge.

The techniques described above could allow for the identification of homologous cell types, as well as taxon-specific cell types. Such an example is the homology between neo-endometrial stromal fibroblasts (neo-ESFs), which are found in eutherian mammals, and paleo-ESFs found in marsupial mammals. Neo-ESFs are evolutionarily related to decidual stromal cells, which are unique to eutherian mammals and are important for the immune tolerance following embryo implantation [42]. How do taxon-specific cell types, such as decidual stromal cells, arise during development? Were they gained in one lineage or were they lost in the other? What are the molecular mechanisms that led to their evolution?

Devo: how to define developmental trajectories

Neuronal cell type diversity is established during development. To recognize the potential for the generation of a new cell type during evolution ("cell speciation"), it is important to know the cell lineage of the tissue of interest in different species to then be able to discover differences in these lineages. Extensive research in model organisms such as worms, flies and mice, has provided a significant breadth of information regarding the development of neuronal tissues. In the absence of such information in an animal of interest, one can use a number of different recently developed techniques that rely on sequential CRISPR-generated mutations and single cell sequencing and allow the reconstruction of cell lineages during development [43–46].

scGESTALT [44], ScarTrace [46], and LINNAEUS [45] rely on the use of CRISPR for generating different marks in multiple target loci that have been integrated in the genome. The inherent variability of the targeting and repair process introduces different mutations at these loci in different branches of the cell lineage. The mutated targets can then be read in adult cells alongside the cell-specific transcriptome using single cell sequencing; the transcriptome allows for the identification of the cell type, while the specific CRISPR-induced mutations are used to reconstruct the lineage tree, in ways similar to the reconstruction of phylogenetic trees based on specific DNA sequences. This has enabled the reconstruction of neuronal lineages in zebrafish, both in larva and adult brains. Moreover, the ease-of-use of CRISPR and efficiency in diverse species [47] provides the opportunity to apply such lineage tracing methods beyond the established model organisms.

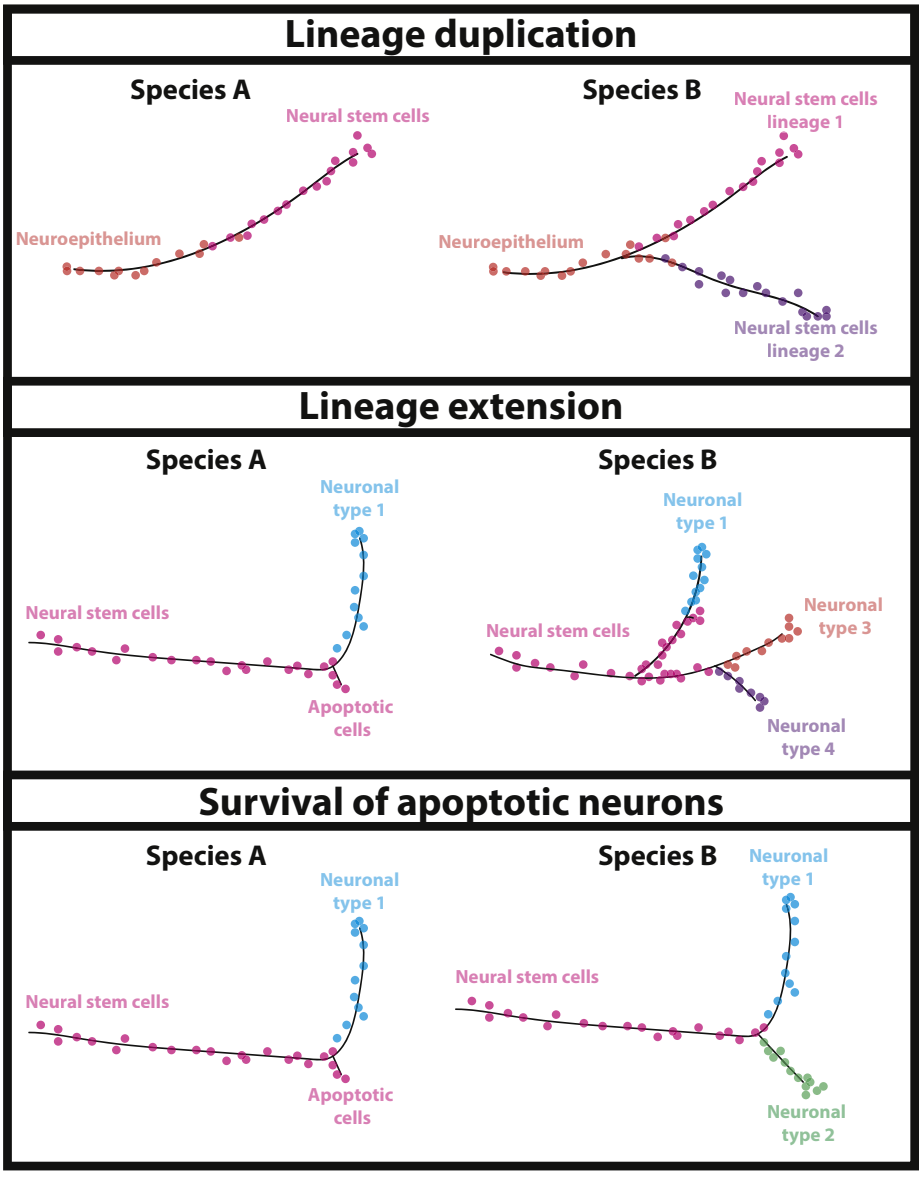
Developmental cell lineages are extremely useful to understand the development of cell types, but coupling

them with the knowledge of the molecules that drive the differentiation of sister cells after each division is necessary to understand how each cell type is generated. A large number of trajectory inference (TI) methods have been developed over the last 5 years to probe developmental trajectories, and predict the critical genes at each bifurcation along the trajectory [6–13].

TI methods typically consist of a dimensionality reduction step and a trajectory-building step. The first step performs dimensionality reduction (PCA, ICA, t-SNE, and diffusion maps), clustering, and/or graph building to represent the cells in a reduced feature space, while maintaining the main characteristics of the data. In the second step, the cells are ordered along the simplified space in order to find a path through a series of nodes. Depending on the algorithm, the user may input different priors such as the initial cell or node or the topology of the trajectory [7].

Although most of the developed algorithms are able to handle multiple bifurcations, it is clear that the more complex the tissue of interest, the harder the reconstruction of the developmental trajectories of its

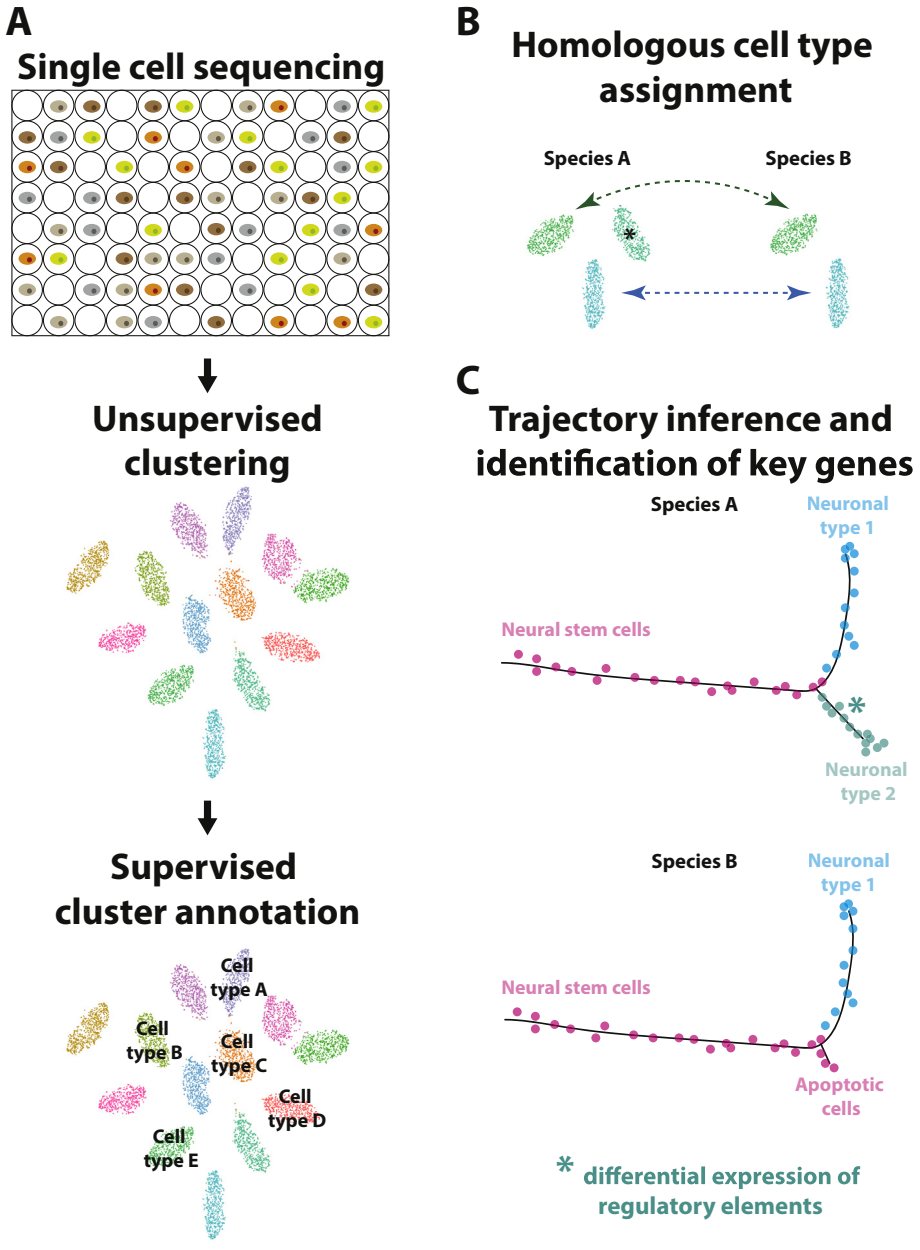
Figure 3



Comparison of developmental trajectories between species. After inferring developmental trajectories for the cell types of interest in two different species, one can compare these trajectories to identify the developmental mechanisms that led to the evolution of the new cell type. Based on the developmental trajectory, one can distinguish between cases of lineage duplication and extension, as well as instances where cells that undergo apoptosis in one species survive in the other, and define key splits in the trajectory of evolutionary related cells. Studying the differential expression of genes along the trajectory, one can identify putative transcription factors that differ between species and may have been involved in the key splits.

constituent cells. Neuronal tissues are among the most complex ones, consisting of a great variety of neuronal cell types. While its complexity is what makes the neuronal tissue attractive for studying how diversity develops and evolves, it is this same complexity that makes it extremely difficult to study. Improving the depth of the sequencing in single cells and the number of available single cell transcriptomes are two factors that can facilitate the reconstruction of reliable developmental trajectories. Moreover, new developmental trajectory algorithms are being developed to accommodate leaps in the development of single cell sequencing technology and the constantly increasing amount of available data.

Figure 4



Overview of technologies and algorithms to probe neuronal cell type evolution. To understand how neuronal cell types evolve, one can combine available single-cell sequencing techniques with powerful algorithms: A) Use massive parallel sequencing technologies to sequence every single cell in the tissue of interest in different species, clustering algorithms to group single cells in transcriptionally similar clusters and then annotate the clusters based on the expression of specific markers. B) Assign homologous cell types between different species (arrows) and identify cell types that are unique in one of them (asterisk). C) Draw developmental trajectories of species-specific cell types and their evolutionary sister cells, compare the trajectories between the two species, and identify key genes that may have played a causal role in the evolution of these species-specific cell types. These genes can be then interrogated for their role using CRISPR driven knock-outs.

Once the developmental trajectories of a tissue in different species are defined, it becomes possible to figure out how cell types that are unique in one animal evolved. Differences in the developmental trajectories can pinpoint key splits in the trajectory of evolutionary related cells, indicate the mechanisms that led to the evolution of new cell types, and suggest transcription factors that differ between species and may have been involved in this split (Figure 3). But what is the significance of these regulators? How do they contribute to the generation of a new cell type? What are the genetic changes that led to the evolution of a new cell type?

CRISPR-induced mutations present the opportunity to test such regulators. Once the regulator that allows for the differentiation of a newly evolved cell type from its evolutionary related cell type is determined, one can look for genomic changes in its cis elements that may have changed its expression. One can then use CRISPR to exchange enhancers and try to force the generation of this cell type in a species where it doesn't exist. This opens up other questions (e.g. the identification of cis and trans elements that influence the expression of genes in specific cell types), which can be addressed using available single cell chromatin accessibility techniques [48,49], and can give us a holistic understanding of how a new neuronal cell type evolved.

Conclusions

We present here how new single cell sequencing technologies and available algorithms can be used to probe neuronal cell type evolution in three steps (Figure 4): a) single cell sequencing of the same tissue in two different species, clustering, and cluster annotation, b) identification of homologous cell types, as well as cell types that are unique in one species, c) trajectory inference for the development of these cell types, comparison of the trajectories in the two species, and identification and validation of candidate transcription factors that may be causing these different trajectories. This will give us the opportunity to address evo-devo questions in a thorough manner, switching from candidate genes to unbiased large-scale sequencing and from model to non-model organisms. As discussed here, important challenges remain, which will soon be possible to overcome with the fast improvements of single-cell sequencing technologies and computational algorithms. Cells are the product of their evolutionary and developmental history, which is reflected in their transcriptome. By reading their history, we will be able to reverse-engineer it to guide successful *in vitro* differentiation of pluripotent stem cells towards specific neuronal types.

Conflict of interest

Nothing declared.

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- •• of outstanding interest

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This paper uses single-cell combinatorial indexing assay for transposase accessible chromatin with sequencing (sci-ATAC-seq) to assess chromatin accessibility changes in the Drosophila nuclei during early embryonic development (2–12 h) and identify tissue-specific regulatory elements.