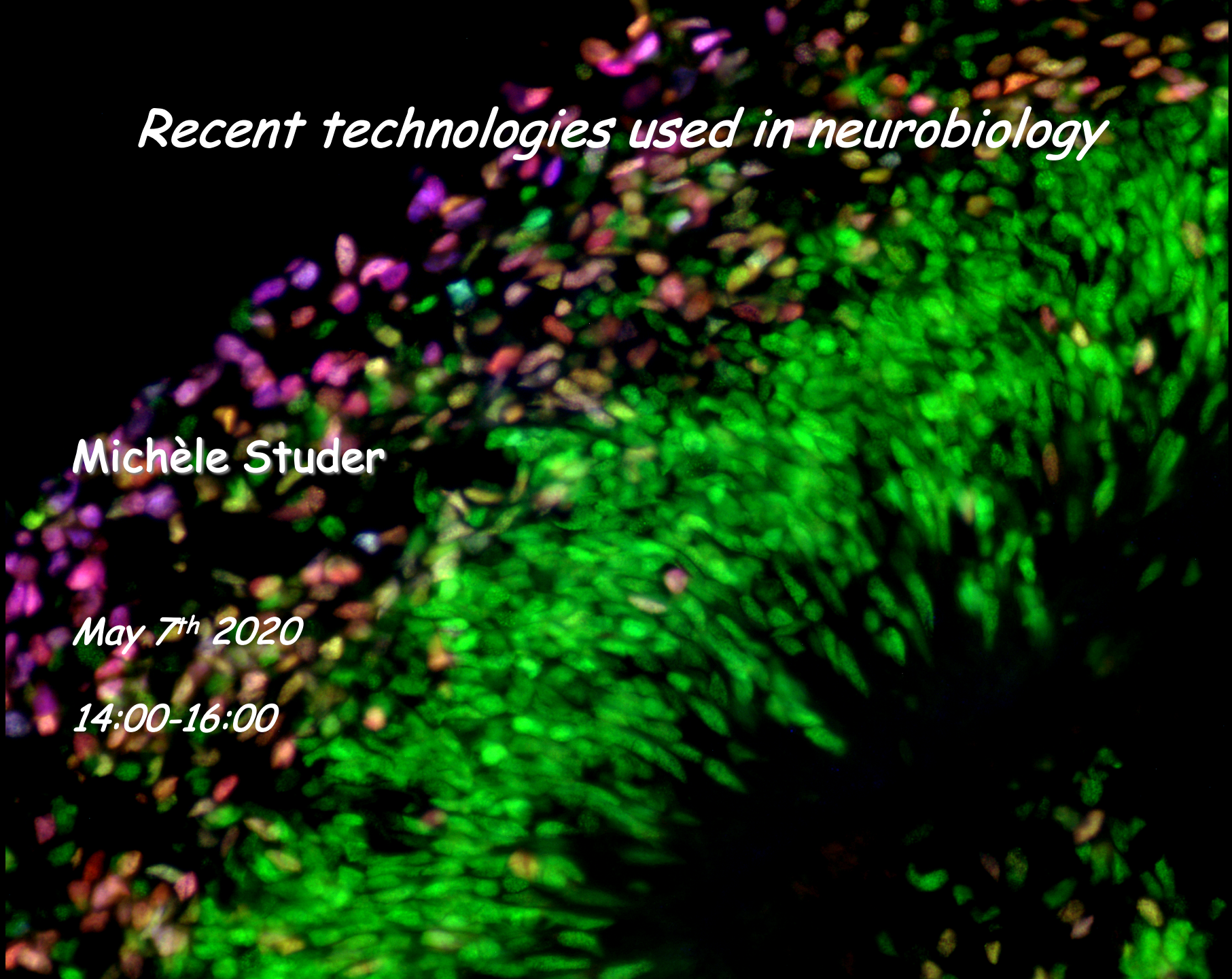

Recent technologies used in neurobiology
Michèle Studer
May 7th 2020
14:00-16:00

## *Some of the most recent and used technologies in modern neurobiology/neuroscience*

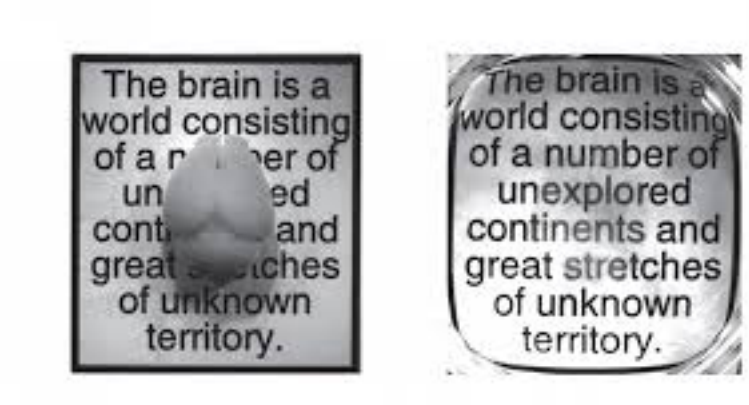
1. *3D whole brain imaging;*
2. *Rabies virus tracing and optogenetics;*
3. *scRNAseq & cell lineage tracking;*
4. *IPSCS & brain organoids;*
5. *In vivo & in vitro reprogramming.*

# *3D whole brain imaging*

## 1. Tissue clearing and imaging

**CLARITY:**
- acrylamide-based protocol making the brain tissue transparent;
- allows intact-tissue imaging of long-range projections, local circuit wiring, cellular relationships, subcellular structures, protein complexes, nucleic acids and neurotransmitters;
- developed by Kwanghun Chung and Karl Deisseroth at the Standford University School of Medicine (Nature 2013).

https://youtu.be/LJ4PA1Gkhkg



# Main Steps Of Solvent-Based Tissue Clearing Techniques

1. DEHYDRATION: reduces scattering of the light by packing together individual particles

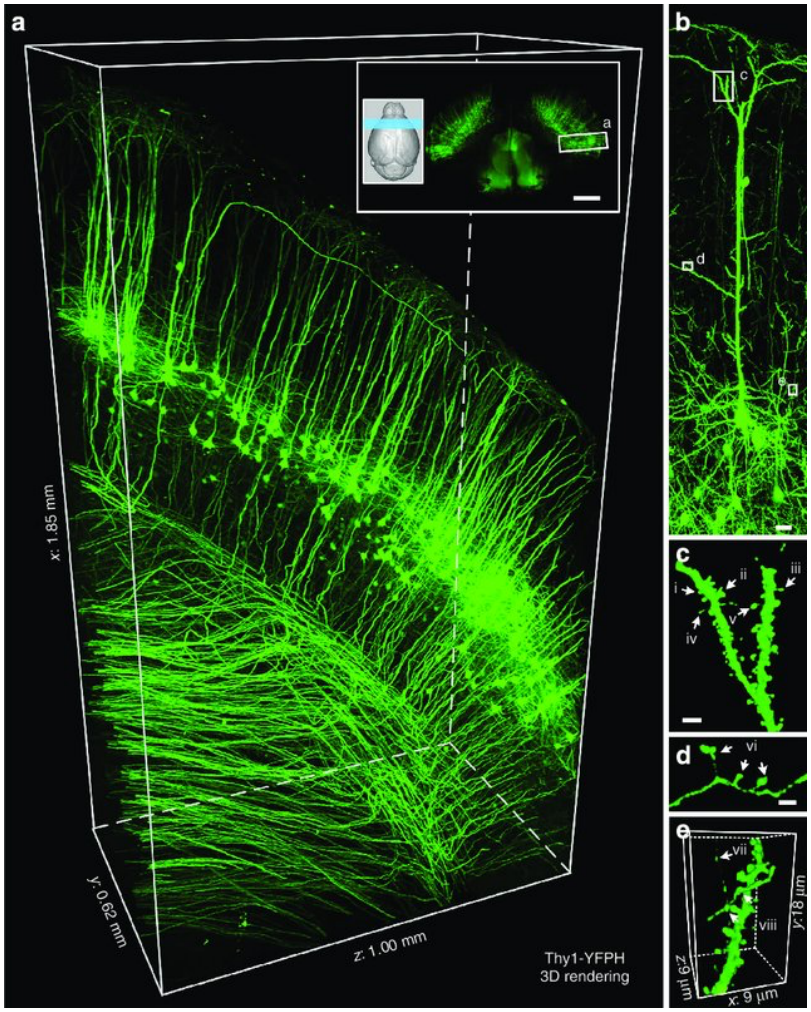
2. LIPID REMOVAL: reduces energy absorbance

3. IMMERSION IN CHEMICAL CLEARING AGENTS: with refractive index similar to proteins (refractive index matching).

# Comparison of Tissue Clearing Techniques

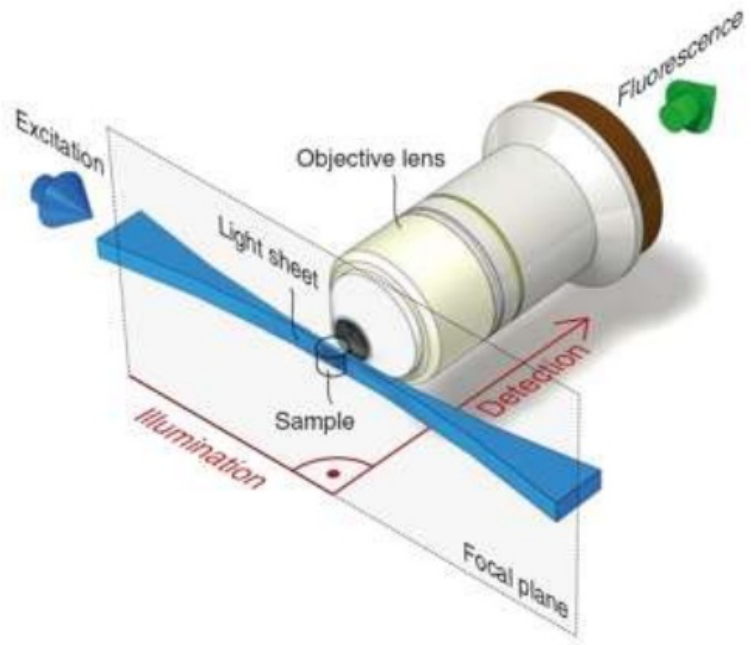
| | Mouse stages | | Immunostaining | Clearing | Endogenous Fluorescence | Tissue shrinkage |
|---|---|---|---|---|---|---|
| | Embryonic stages | Postnatal | | | | |
| ***Solvents*** | | | | | | |
| **3DISCO** | Entire embryo | Isolated organs | Before clearing | 1d | - | Up to 50% |
| **iDISCO+** | Entire embryo | Isolated organs | Before clearing | 2d | - | Up to 10% |
| **uDISCO** | - | Entire animal | Before clearing | 3d – 1week | Yes | Up to 40% |
| ***Reagents*** | | | | | | |
| **CLARITY** | Entire embryo | Isolated organs | After clearing | 5d -2 months | Yes | variable |
| **PACT** | - | 3mm slide | After clearing | 2 weeks | Yes | variable |
| **PARS** | - | Entire animal | After clearing | 4d -2wks | Yes | variable |

*Adapted from Vigourox et al., 2017*




https://youtu.be/D84wBVDX1No

# Light-sheet microscopy (LSM)




from J. Huisken and D. Y. Stainier (Development, 2009)

**Key advantages:**

Optical sectioning with wide field detection scheme

↓

Fast high resolution 3D imaging

Optical sectioning with low-NA optics (having longer WD)

↓

Imaging of large specimens without sample sectioning.

Only the observed plane is illuminated

↓

Reduced photobleaching.

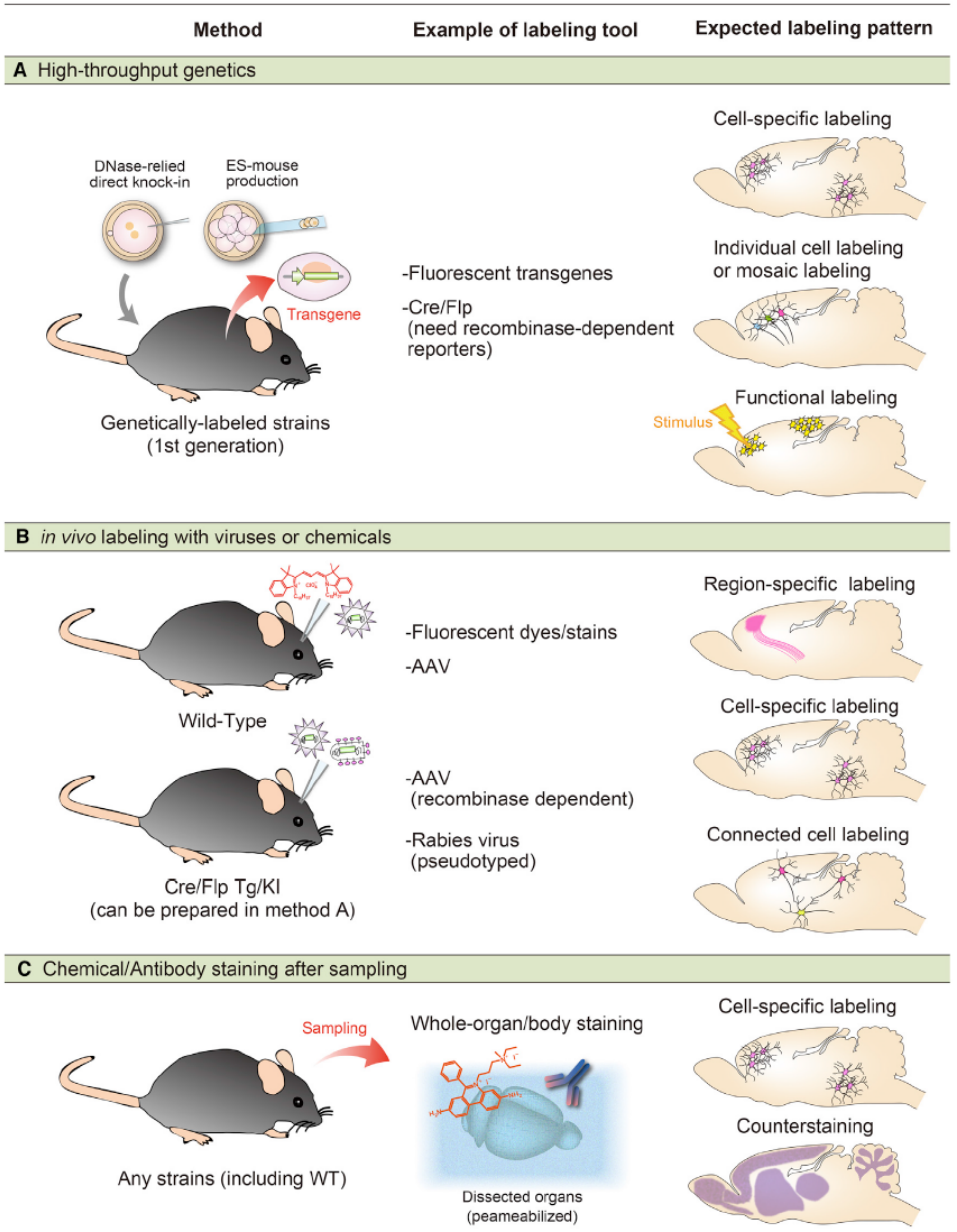
## Advantages

- Quick acquisition of complete histological information in large tissues
- Easy
- 3DISCO and iDISCO very fast to perform vs. imaging tissue sections
- 3DISCO and iDISCO are compatible with the majority of tested antibodies

## Limitations

- Can only be used on fixed tissues
- Optical clearing dissolves the lipid structures, so the cleared tissue cannot be further studied with electron microscopy
- Not compatible with lipophilic tracing dyes
- Some fluorescent proteins (i.e. GFP and YFP) are less stable than others in clearing solutions

# Tissue-clearing applications and volume-imaging in Neuroscience



Mini-Symposium

# Whole-Brain Analysis of Cells and Circuits by Tissue Clearing and Light-Sheet Microscopy

**Tomoyuki Mano,[1,2]* Alexandre Albanese,[4]* Hans-Ulrich Dodt,[8,9] Ali Erturk,[10,11,12] Viviana Gradinaru,[13] Jennifer B. Treweek,[13,14] Atsushi Miyawaki,[15,16] Kwanghun Chung,[4,5,6,7,17] and Hiroki R. Ueda[2,3,18]**

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**In this photo essay, we present a sampling of technologies from laboratories at the forefront of whole-brain clearing and imaging for high-resolution analysis of cell populations and neuronal circuits. The data presented here were provided for the eponymous Mini-Symposium presented at the Society for Neuroscience's 2018 annual meeting.**

## Introduction

Microscopic analysis of tissues reveals that intricate organization of cells underlies biological function. Tissues are not translucent against visible light, so it is impossible to image far beyond the surface. Thus, conventional tissue imaging applies a microtome to cut samples into thin sections before staining with dyes and antibodies to visualize cells. Inferring 3D structure from thin sections is often problematic, and requires minimal sample distortion and precision alignment of serial sections.

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The Dodt laboratory thanks Dr. Saiedeh Saghafi and Dr. Klaus Becker for aspheric optics-based LSFM work, and the Austrian science funding agency FWF and the German Hertie foundation for support; the Ertürk laboratory thanks Ruiyao Cai for uDISCO figure and Synergy Excellence Cluster Munich (SyNergy), Fritz Thyssen Stiftung, and DFG for support; the Miyawaki laboratory thanks Dr. Hiroshi Hama for Sca*l*eS figure and Grant-in-Aid for Scientific Research on Priority Areas (JSPS KAKENHI), the Human Frontier Science Program, and the Brain Mapping by Integrated Neurotechnologies for Disease Studies (Brain/MINDS) for support; the Ueda laboratory thanks Tatsuya Murakami for offering picture materials for CUBIC figure, and Brain/MINDS (AMED/MEXT), the Basic Science and Platform Technology Program for Innovative Biological Medicine (AMED/MEXT)and Grant-in-Aid for Scientific Research (S) (JSPS KAKENHI) for support; the Chung laboratory thanks The Packard Award, the McKnight Foundation and the NIH (1-DP2-ES027992) for support; the Gradinaru laboratory thanks Sripriya Ravindra Kumar, Gerard M. Coughlin, Rosemary Challis, and Collin Challis for CREATE and VAST images, Min Jee Jang for HCR images, and J. Ryan Cho for GCaMP imaging, and the NIH BRAIN Initiative and NIH Director's Office and NSF Neuronex for support.

The authors declare no competing financial interests.

*T.M. and A.A. contributed equally to this work.

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https://doi.org/10.1523/JNEUROSCI.1677-18.2018

 0270-6474/18/389330-08$15.00/0

The analysis of large 3D volumes is necessary for mapping the connections of far-reaching neurons inside the brain and determining the nature of cellular interactions underlying proper function and behavior. In recent years, several techniques have emerged to achieve optical transparency and enable high-resolution microscopy of thick tissue sections and whole organs. These techniques use different strategies to reduce light scattering in tissues and improve image sharpness.

Scattering occurs in tissues due to light's heterogeneous interaction with different molecules, subcellular structures, membranes, and cell populations inside the tissue. For example, the interface between a cell's lipid membrane and the cytoplasm causes a significant drop in refractive index (RI). Heterogeneity at the scale of molecules, cells, and tissues contribute to light scattering and requires homogenization via tissue clearing to increase overall light penetration (Tainaka et al., 2016; Treweek and Gradinaru, 2016). Combining tissue clearing with light-sheet fluorescence microscopy (LSFM) has paved the road for current whole-brain imaging by eliminating out-of-focus excitation (hence reduced background level and greatly preventing photobleaching), and by accelerating image acquisition.

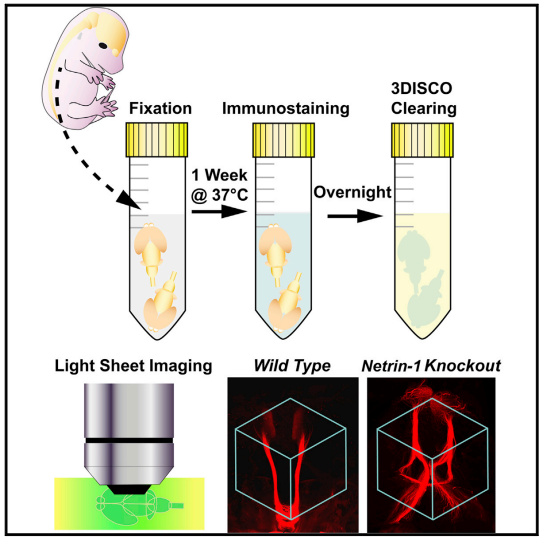
Tissue clearing originated in the early 20th century, when Werner Spalteholz experimented with high RI organic solvents (Spalteholz, 1914). Organic solvent-based clearing homogenizes a tissue's RI by the removal of highly scattering lipids and the displacement of water by high RI solvents. Building on this approach, Dodt's group revitalized organic solvent-based tissue clearing for the modern era of neuroscience by coupling it with a new optical imaging method that

Report

Cell Reports

# A Simple Method for 3D Analysis of Immunolabeled Axonal Tracts in a Transparent Nervous System

## Graphical Abstract




## Highlights

Immunostaining and 3DISCO clearing: a powerful method for studying brain connections

3D analysis of axon guidance defects in midline mutant mice

Unexpected roles for Slits and Netrin-1 in fasciculus retroflexus development

## Authors

Morgane Belle, David Godefroy, ..., Frank Bradke, Alain Chédotal

## Correspondence

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## In Brief

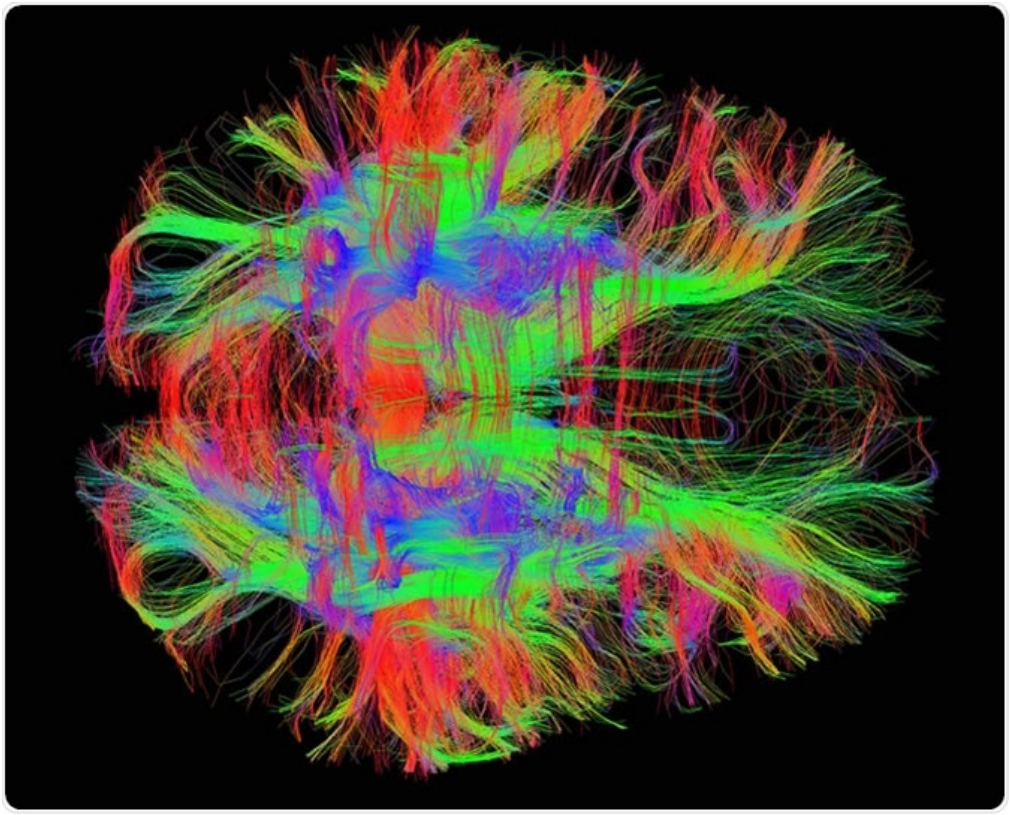
Clearing techniques have recently been developed to look at mouse brains, but they are complex and expensive. Belle et al. now describe a simple procedure that combines immunolabeling, solvent-based clearing, and light-sheet fluorescence microscopy. This technique allows large-scale screening of axon guidance defects and other developmental disorders in mutant mice.

Belle et al., 2014, Cell Reports 9, 1191–1201
November 20, 2014 ©2014 The Authors
http://dx.doi.org/10.1016/j.celrep.2014.10.037

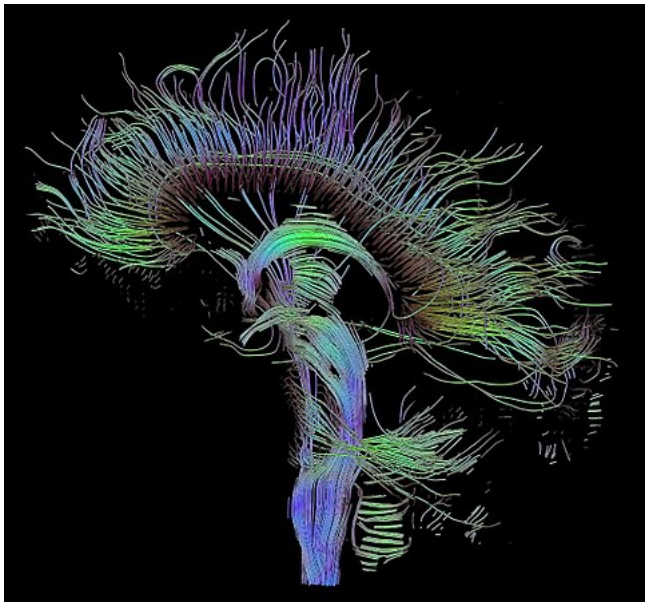
# *3D whole brain imaging*

## 2. Diffusion tensor imaging (DTI)

DTI is a newly developed magnetic resonance imaging (MRI) technique that analyses the anatomy of nerve cells and a complex neuronal network of the brain. It is extensively used to map white matter tractography in brains.



*White matter fiber tracts in the adult human brain*



*Cerebral Cortex*, November 2015;25: 4628–4637

**doi: 10.1093/cercor/bhv121**
Advance Access Publication Date: 5 June 2015
Original Article

ORIGINAL ARTICLE

# A Diffusion MRI Tractography Connectome of the Mouse Brain and Comparison with Neuronal Tracer Data

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## Abstract

Interest in structural brain connectivity has grown with the understanding that abnormal neural connections may play a role in neurologic and psychiatric diseases. Small animal connectivity mapping techniques are particularly important for identifying aberrant connectivity in disease models. Diffusion magnetic resonance imaging tractography can provide nondestructive, 3D, brain-wide connectivity maps, but has historically been limited by low spatial resolution, low signal-to-noise ratio, and the difficulty in estimating multiple fiber orientations within a single image voxel. Small animal diffusion tractography can be substantially improved through the combination of ex vivo MRI with exogenous contrast agents, advanced diffusion acquisition and reconstruction techniques, and probabilistic fiber tracking. Here, we present a comprehensive, probabilistic tractography connectome of the mouse brain at microscopic resolution, and a comparison of these data with a neuronal tracer-based connectivity data from the Allen Brain Atlas. This work serves as a reference database for future tractography studies in the mouse brain, and demonstrates the fundamental differences between tractography and neuronal tracer data.

**Key words:** connectome, magnetic resonance imaging, mouse, neuroanatomy, tractography

## Introduction

Brain connectivity mapping has emerged as a major focus of neuroscience research, in part due to the recognition that altered neural connectivity may contribute to a number of neurologic and psychiatric diseases (Konrad and Eickhoff 2010; Lo et al. 2010; Skudlarski et al. 2010; Xue et al. 2014). Several techniques have been used to explore structural brain connectivity in animal models including anterograde/retrograde tracer studies (Swanson 1982; Oh et al. 2014), two-photon tomography (Ragan et al. 2012), diffusion magnetic resonance imaging (MRI) tractography (Mori et al. 1999), and polarized light imaging (Larsen et al. 2007; Axer et al. 2011). Neuronal tracer studies have become the de facto gold standard for brain connectivity mapping because of their high sensitivity and specificity; however, these studies are limited by the requirement for stereotaxic tracer injections, 2D (slice) imaging, and the inability to study multiple pathways within a single brain.

Diffusion MRI tractography—the 3D tracing of water diffusion pathways measured by MRI—offers a noninvasive method for brain connectivity mapping. Tractography is generated from anatomically defined seed regions of arbitrary size (above image resolution), shape, and number, allowing a complete connectivity map, or connectome, to be developed from a single brain. Despite these benefits, diffusion tractography has historically been limited to the study of gross connectivity between distant anatomic regions, and generally fails to accurately represent mesoscale brain connectivity (i.e., ~100 µm resolution) (Oh et al. 2014). This limitation is primarily due to the low spatial resolution and low signal-to-noise ratio (SNR) of diffusion MRI, and

nature COMMUNICATIONS

ARTICLE

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# Microtubule-associated protein 6 mediates neuronal connectivity through Semaphorin 3E-dependent signalling for axonal growth

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Structural microtubule associated proteins (MAPs) stabilize microtubules, a property that was thought to be essential for development, maintenance and function of neuronal circuits. However, deletion of the structural MAPs in mice does not lead to major neurodevelopment defects. Here we demonstrate a role for MAP6 in brain wiring that is independent of microtubule binding. We find that MAP6 deletion disrupts brain connectivity and is associated with a lack of post-commissural fornix fibres. MAP6 contributes to fornix development by regulating axonal elongation induced by Semaphorin 3E. We show that MAP6 acts downstream of receptor activation through a mechanism that requires a proline-rich domain distinct from its microtubule-stabilizing domains. We also show that MAP6 directly binds to SH3 domain proteins known to be involved in neurite extension and semaphorin function. We conclude that MAP6 is critical to interface guidance molecules with intracellular signalling effectors during the development of cerebral axon tracts.

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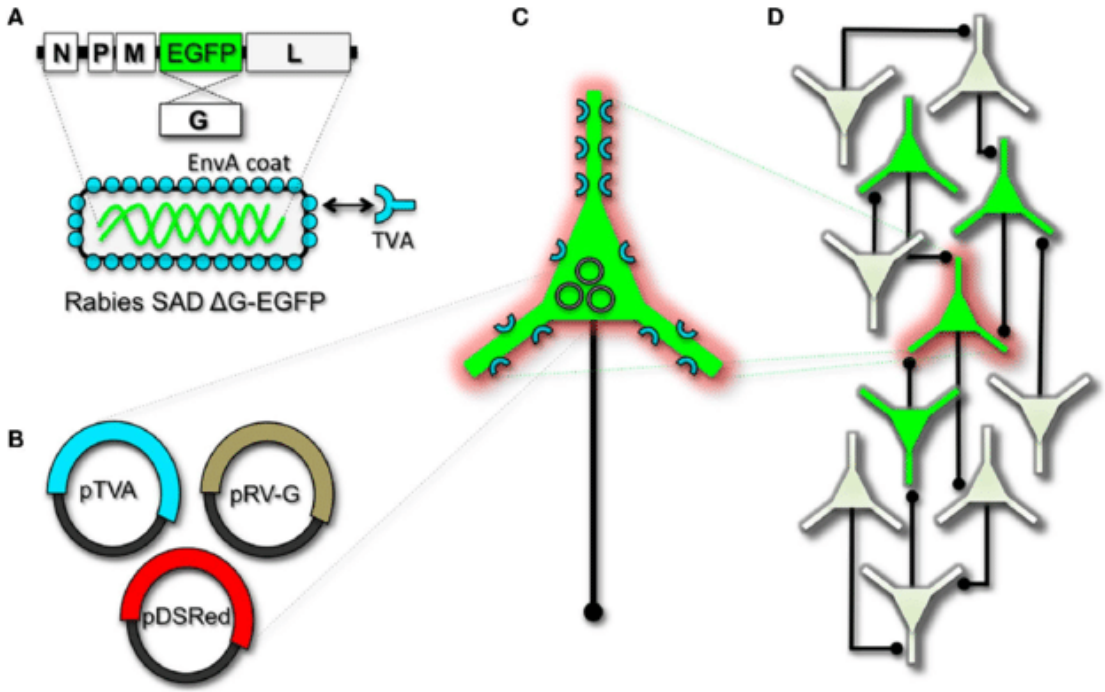
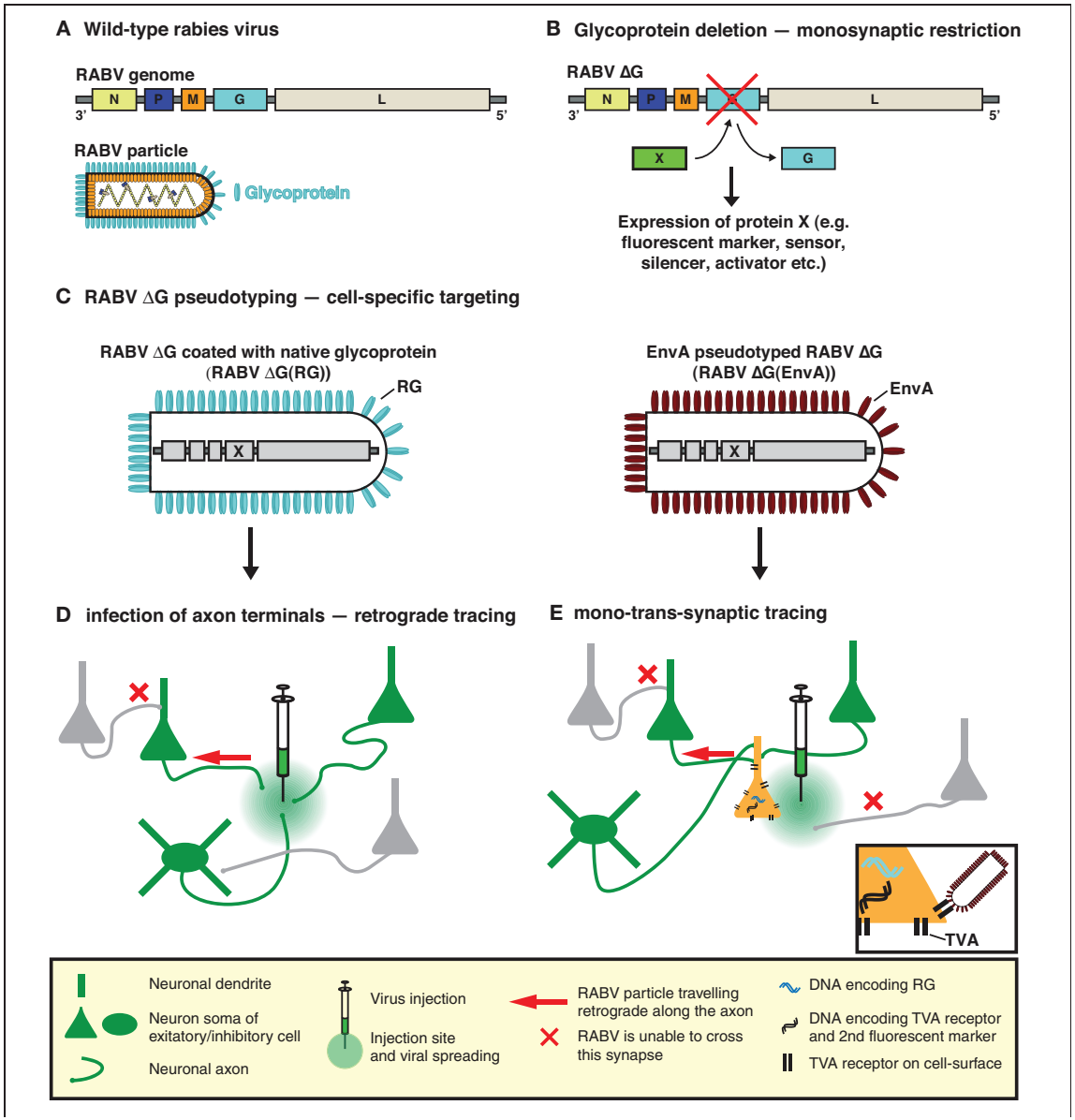
# *Rabies virus for transneuronal tracing*

- Recombinant rabies virus (RABV) are synapse-specific trans-neuronal tracer
- Pseudotyped delta G rabies viruses (ΔG RABV) map direct synaptic connections
- In combination with optogenetics and in vivo imaging methods they are very useful tools in understanding connectivity and function of the nervous system

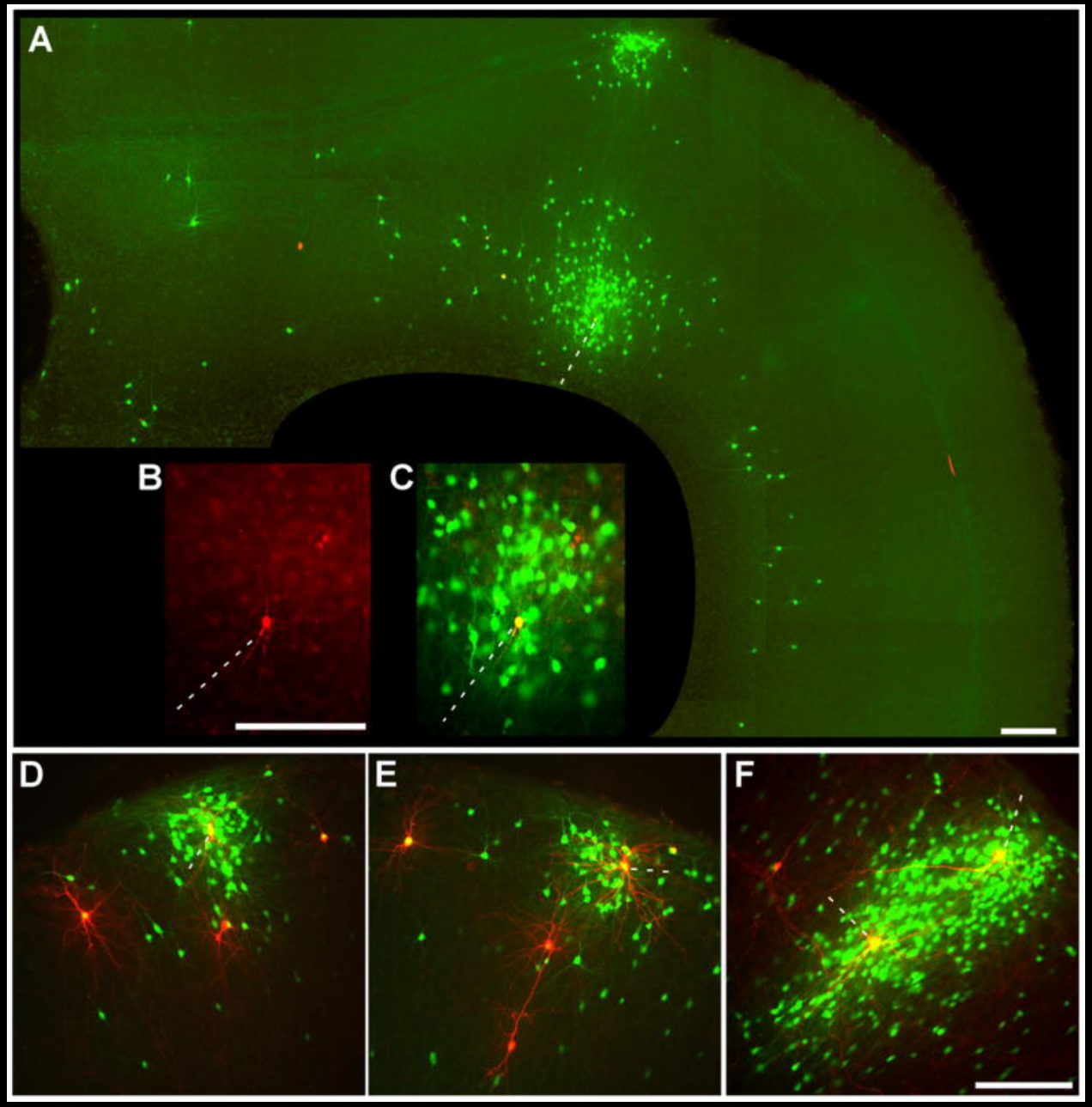



*Wickersham et al., 2007*

# RABV—the trans-synaptic tracing toolbox




Ginger et al., 2013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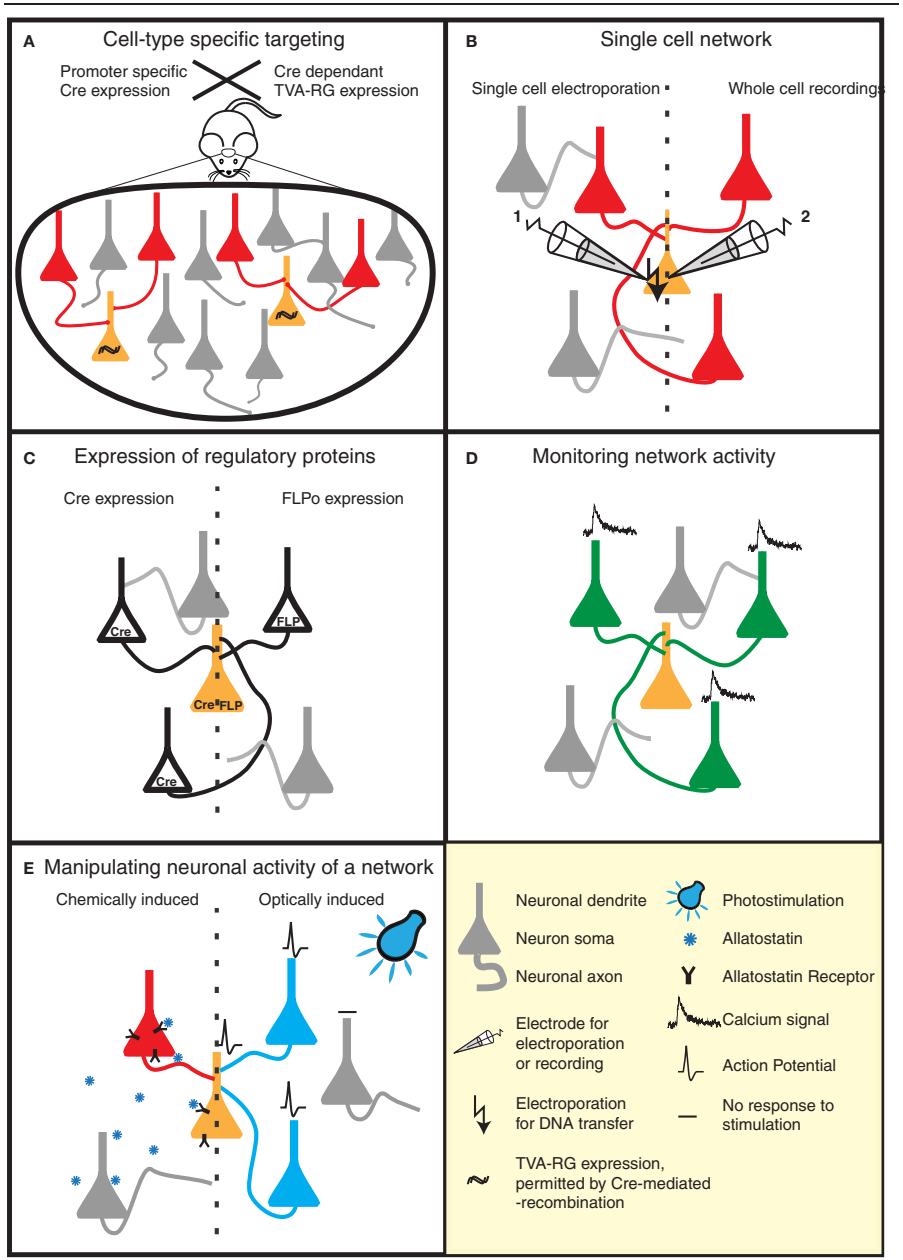
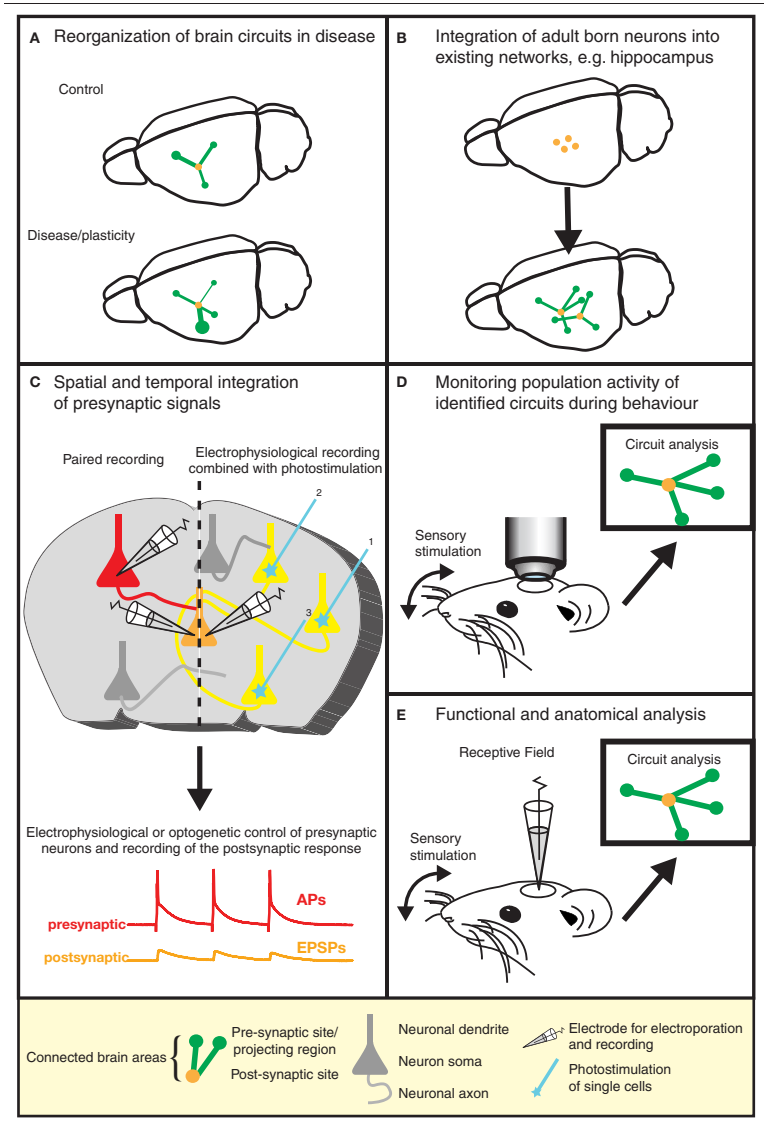
# Strategies to reveal defined neural circuits, individual neurons or specific cell types



# Gaining insight into brain function by dissecting neuronal circuits

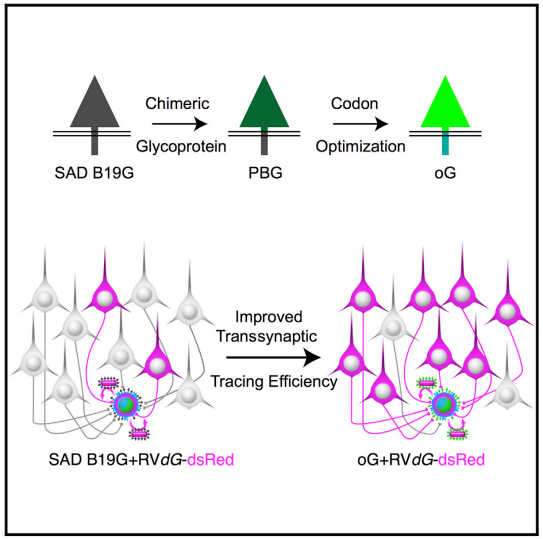


Cell Reports



# Improved Monosynaptic Neural Circuit Tracing Using Engineered Rabies Virus Glycoproteins

## Graphical Abstract




## Authors

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## In Brief

Glycoprotein-deleted rabies virus is widely used to trace neural circuits, but it labels only a fraction of all presynaptic neurons. Kim et al. provide a simple method to increase transsynaptic tracing efficiency by adopting the engineered and optimized glycoprotein (oG).

## Highlights

- Newly engineered glycoproteins improve monosynaptic rabies tracing
- Optimized glycoprotein (oG) increases tracing efficiency up to 20-fold

Kim et al., 2016, Cell Reports *15*, 692–699
April 26, 2016 
http://dx.doi.org/10.1016/j.celrep.2016.03.067

CellPress

PRESYNAPTIC NETWORKS

# Single-cell–initiated monosynaptic tracing reveals layer-specific cortical network modules

Adrian Wertz,[1]* Stuart Trenholm,[1]* Keisuke Yonehara,[1] Daniel Hillier,[1] Zoltan Raics,[1] Marcus Leinweber,[1] Gergely Szalay,[2] Alexander Ghanem,[3] Georg Keller,[1] Balázs Rózsa,[2] Karl-Klaus Conzelmann,[3] Botond Roska[1,4]†

**Individual cortical neurons can selectively respond to specific environmental features, such as visual motion or faces. How this relates to the selectivity of the presynaptic network across cortical layers remains unclear. We used single-cell–initiated, monosynaptically restricted retrograde transsynaptic tracing with rabies viruses expressing GCaMP6s to image, in vivo, the visual motion–evoked activity of individual layer 2/3 pyramidal neurons and their presynaptic networks across layers in mouse primary visual cortex. Neurons within each layer exhibited similar motion direction preferences, forming layer-specific functional modules. In one-third of the networks, the layer modules were locked to the direction preference of the postsynaptic neuron, whereas for other networks the direction preference varied by layer. Thus, there exist feature-locked and feature-variant cortical networks.**

In the cortex, many neurons selectively respond to distinct environmental features such as image motion in a specific direction or orientation (*1*, *2*), the spatial position of the animal (*3*), or a specific part of a face (*4*). Each cortical neuron receives input from hundreds of nearby neighbors. Understanding the feature preference of the cortical neurons that provide input to a neuron with an identified feature preference could help us to understand how selectivity emerges and how cortical circuits are organized.

Within layer 2/3 of mouse primary visual cortex (V1), there is a close relation between orientation selectivity and synaptic connectivity (*5*). In contrast, in vivo single-cell recordings from layer 2/3 pyramidal cells in V1 have revealed different degrees of similarity between the preferred orientations at the dendritic input sites and at the cell body (*6*–*8*). The variability of orientation preferences at dendritic input sites could arise from differently tuned inputs from deeper cortical layers. The relation between the feature selectivity of the postsynaptic cell and the feature selectivity and functional organization of the deeper cortical neurons that provide synaptic input to individual layer 2/3 cells is still unknown.

We combined functionalized transsynaptic tracing with two-photon imaging and recorded the visually evoked responses of individual layer 2/3 pyramidal neurons together with their presynaptic neuronal networks across different cortical layers in vivo in mouse V1. Single-cell–initiated monosynaptic tracing (*9*) allows the expression of genetic tools in individual cortical neurons together with their monosynaptically connected presynaptic partners (*10*). This form of tracing is based on the delivery of three plasmids to a single neuron (one expressing a fluorophore, one expressing the avian receptor TVA, and one expressing the rabies virus envelope glycoprotein) and the local infection with the glycoprotein gene–deleted rabies virus coated with envelope-A [(EnvA), the ligand for the TVA receptor]. The EnvA-TVA ligand-receptor interaction restricts rabies virus infection to the TVA-expressing starter cell, and the glycoprotein allows rabies virus to move transsynaptically in the retrograde direction to only those neurons that monosynaptically connect to the starter cell (*9*). Rabies virus can be engineered to express genetically encoded calcium sensors, allowing the activity of the infected neurons to be recorded. The single-cell–initiated tracing system has been used for anatomical studies (*9*, *11*, *12*), but not yet for functional analyses.

Our modified version of single-cell–initiated monosynaptic tracing (*9*) differed in two aspects from previous approaches (Fig. 1A and supplementary materials and methods). First, we electroporated starter cells with four plasmids instead of three; the fourth plasmid, expressing a genetically encoded calcium sensor (GCaMP6s) (*8*), was necessary to record responses from the electroporated starter neuron. Second, we used a new rabies virus variant expressing GCaMP6s (materials and methods), which allowed for monitoring the activity from many presynaptic cells around the starter cell in a region spanning from layer 2/3 to layer 5. In each mouse, we labeled only a single layer 2/3 cell and its presynaptic network (Fig. 1B). We identified V1 using intrinsic in vivo imaging (*13*) (Fig. 1B and fig. S1) or post hoc confocal imaging in fixed brain slices (fig. S2). Soon after electroporation and rabies virus injection, the starter cell exhibited fluorescence (Fig. 1B). All starter cells were pyramidal cells. Next, presynaptic neurons expressing GCaMP6s appeared around the starter cell and increased in number as a function of time, whereas the responsiveness of the starter cell decreased as a function of time (Fig. 1, C and D, and fig. S3). Functional responses from presynaptic cells could be recorded up to ~2 weeks after electroporation, whereas starter cell responses could be recorded for ~1 week.

The total number of cells labeled in a presynaptic circuit, determined post hoc in immunostained brain slices, was 417 ± 74 (ranging from 70 to 846 cells, $n = 9$ presynaptic networks). The most abundant cluster of presynaptic cells surrounded the starter cell and was distributed across cortical layers (332 ± 64 presynaptic cells, ranging from 58 to 729 cells, $n = 9$) (Fig. 1, E to G, and fig. S4) (*14*). Within this local cluster, 82.5 ± 2.4% of cells were pyramidal cells (*15*). Outside of the local cluster of cortical neurons surrounding the starter cell, presynaptic cells were consistently labeled in several other brain regions that provide input to V1 (*14*) (fig. S5).

To determine the visual responses from the starter cell and its presynaptic network in V1, we presented animals with gratings that moved in eight directions and imaged GCaMP6s fluorescence with a two-photon laser scanning microscope from single optical planes with an area of 300 μm by 400 μm, from 40 to 600 μm below the brain surface, at 15- to 20-μm steps (Fig. 2A). Here we present functional data from 17 presynaptic networks connected to single pyramidal cells. From seven of these networks, in which the starter cell was electroporated with the four plasmids, we obtained visual motion responses from both the starter neuron and the presynaptic network. From 10 of the networks, electroporated with the three-plasmid approach (*9*), we obtained recordings from the presynaptic networks but not from the starter cells.

We imaged 98 ± 16 presynaptic cells in each presynaptic network. Nearly half (43 ± 4%) of these cells showed responses to image motion. Responses to motion were quantified using a direction-selective index (DSI) and an orientation-selective index (OSI) (calculated based on the vector sum of responses in all directions) (*16*) (fig. S6 and materials and methods). All presynaptic networks contained both direction- and orientation-selective neurons, and the degree of direction and orientation selectivity varied from neuron to neuron within a given network (Fig. 2, B to D, and fig. S6). Therefore, we analyzed all presynaptic networks for both direction and orientation selectivity.

Neurons within a single presynaptic network could be tuned to similar or different directions and orientations. We quantified the variability

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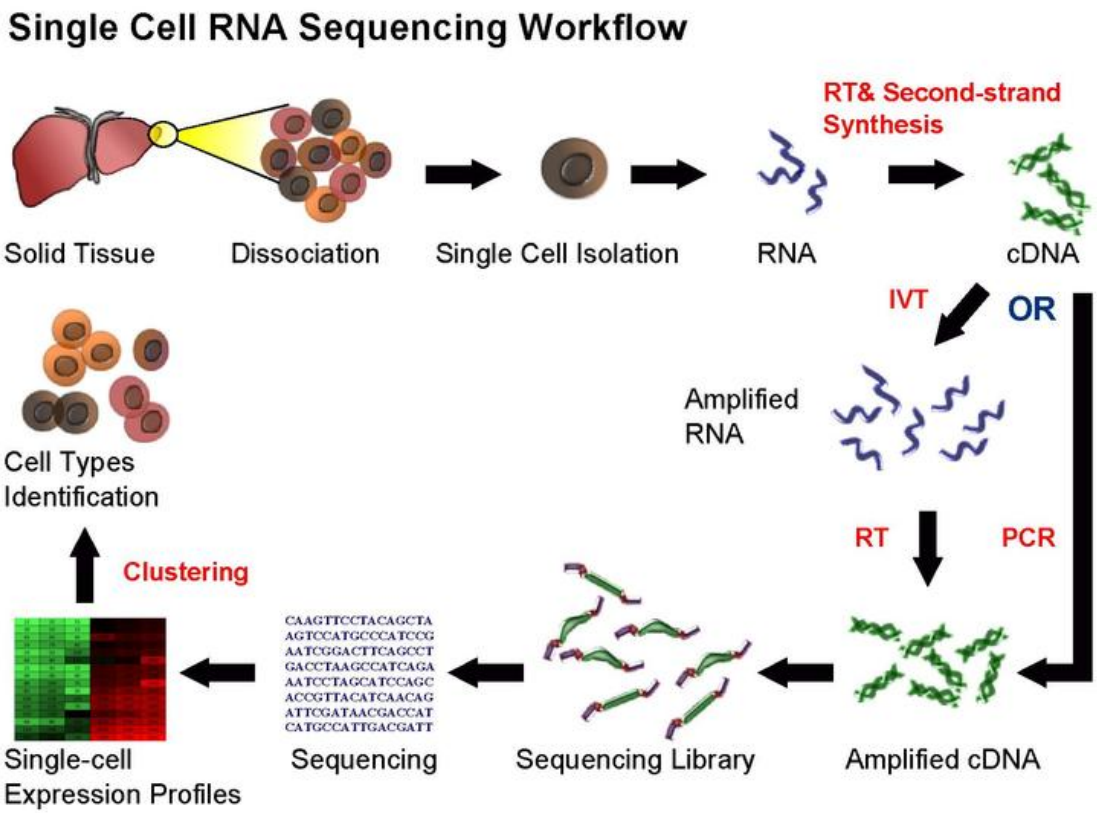
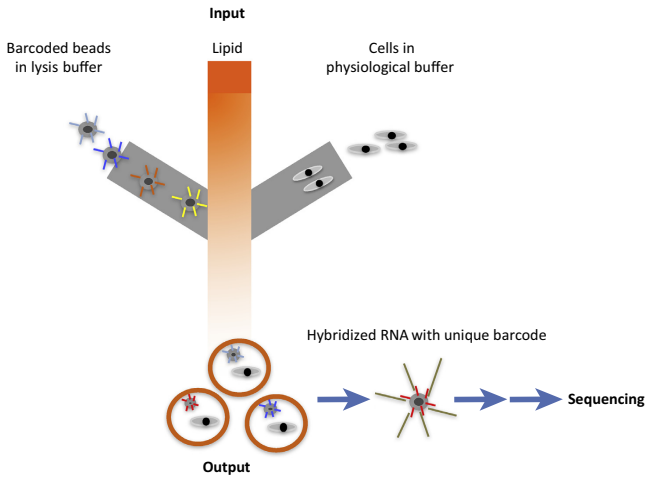
*These authors contributed equally to this work. †Corresponding author. E-mail: botond.roska@fmi.ch

# scRNA-Seq & cell lineage tracking



# Single-cell Sequencing Data Analysis Workflow

**Sequencing**

Read1.fastq.gz

Read2.fastq.gz

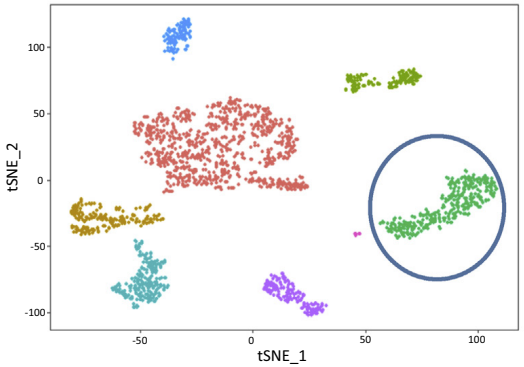
**Extract UMI**

Generate cell–gene expression tables

**Gene expression profile**

| | ACAAAGTCAATN | GACCTCGGCATC | AATGTAAGAACG | CATAAATATCTG | ACTGTGCGATAC |
|---|---|---|---|---|---|
| 0610005C13Rik | 0 | 0 | 0 | 0 | 0 |
| 0610007N19Rik | 0 | 0 | 0 | 0 | 0 |
| 0610007P14Rik | 1 | 0 | 1 | 0 | 0 |
| 0610009B22Rik | 2 | 1 | 1 | 0 | 0 |
| 0610009D07Rik | 2 | 1 | 3 | 2 | 1 |
| 0610009E02Rik | 0 | 0 | 0 | 0 | 0 |
| 0610009L18Rik | 1 | 0 | 0 | 0 | 0 |
| 0610009O20Rik | 1 | 0 | 0 | 1 | 1 |
| 0610010F05Rik | 0 | 0 | 0 | 1 | 0 |
| 0610010K14Rik | 0 | 0 | 0 | 0 | 0 |
| 0610011F06Rik | 4 | 4 | 1 | 10 | 3 |
| 0610030E20Rik | 0 | 0 | 0 | 0 | 0 |

**Cellular landscape (neurons, glia, etc.)**

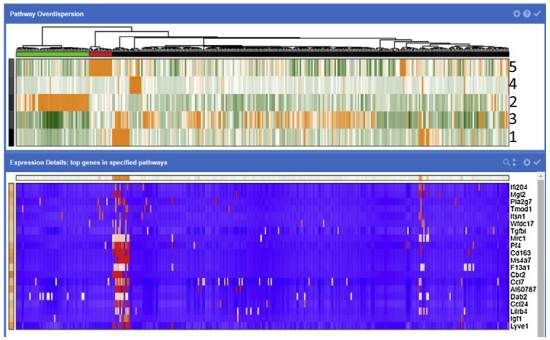



**Cluster cells**

**Experimental group changes (e.g., control vs disease)**

**Subset data**

Singl-cell differential expression and pathway/gene set overdispersion analysis

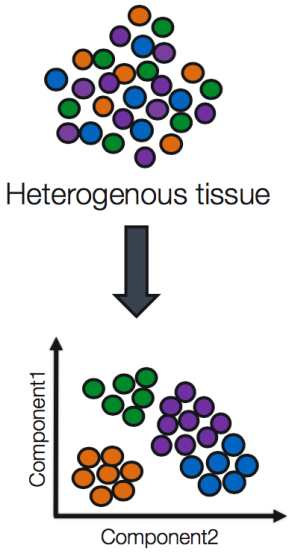



**Novel disease-relevant mechanisms and biomarkers?**

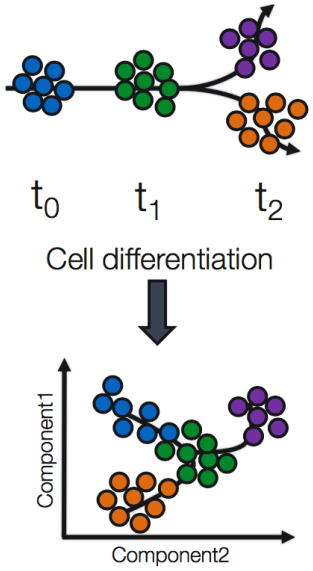
Ofengeim et al., 2017

# Applications of single-cell transcriptome sequencing

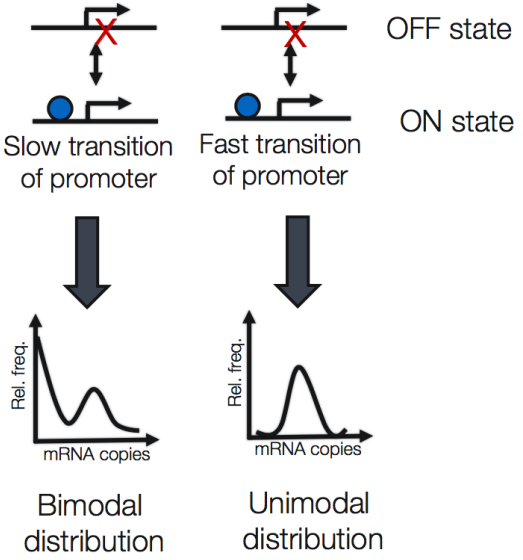
## Studying heterogeneity




## Lineage tracing study

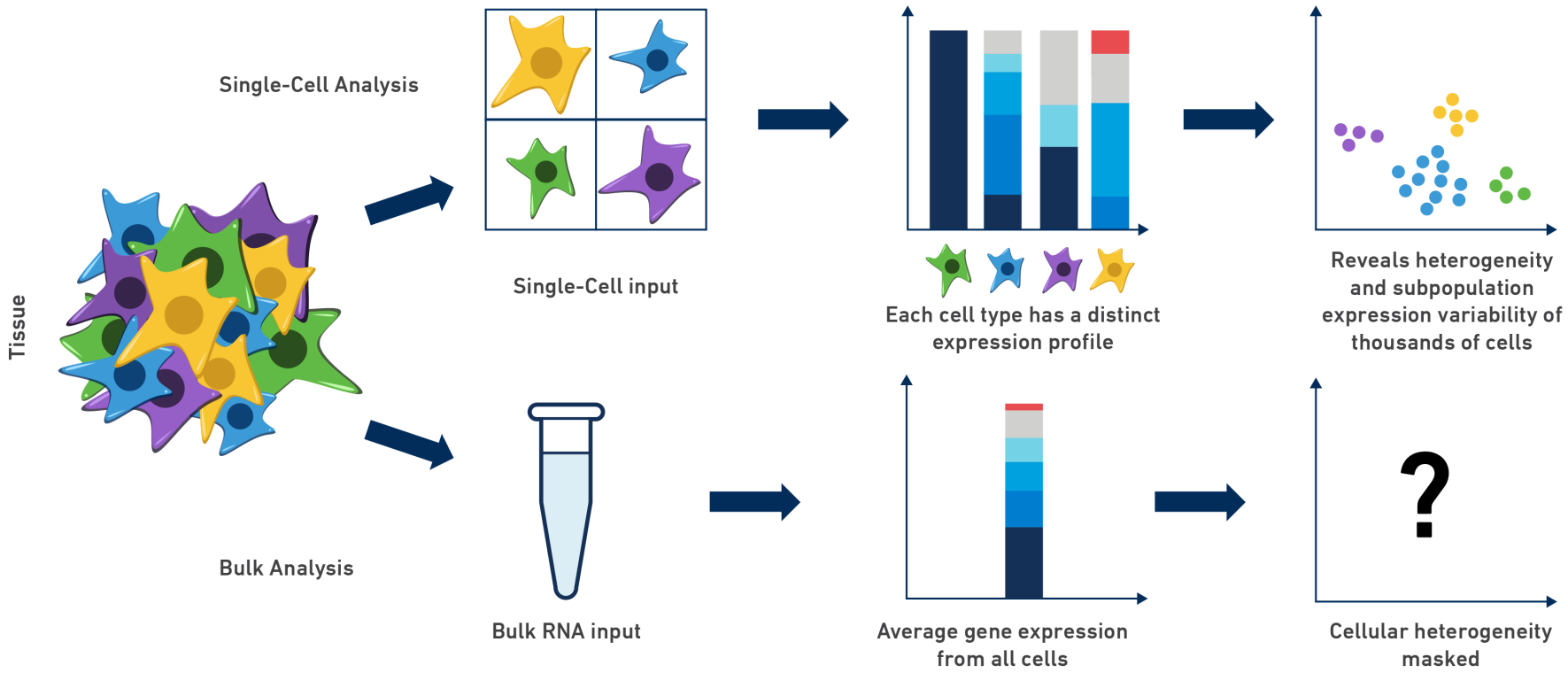



## Stochastic gene expression study




Liu S and Trapnell C. Single-cell transcriptome sequencing: recent advances and remaining challenges, F1000 Research 2016 (doi: 10.12688/f1000research.7223.1)
Junker and van Oudenaarden; Every Cell Is Special: Genome-wide Studies Add a New Dimension to Single-Cell Biology, Cell 2014 (doi: 10.1016/j.cell.2014.02.010)

# Differences between scRNA and bulk RNA sequencing



nature COMMUNICATIONS

ARTICLE

https://doi.org/10.1038/s41467-018-08079-9 OPEN

# Single-cell transcriptomic analysis of mouse neocortical development

Lipin Loo[1], Jeremy M. Simon[1,2,3], Lei Xing[1], Eric S. McCoy[1], Jesse K. Niehaus[1], Jiami Guo[1,2], E.S. Anton[1,2] & Mark J. Zylka[1,2]

The development of the mammalian cerebral cortex depends on careful orchestration of proliferation, maturation, and migration events, ultimately giving rise to a wide variety of neuronal and non-neuronal cell types. To better understand cellular and molecular processes that unfold during late corticogenesis, we perform single-cell RNA-seq on the mouse cerebral cortex at a progenitor driven phase (embryonic day 14.5) and at birth—after neurons from all six cortical layers are born. We identify numerous classes of neurons, progenitors, and glia, their proliferative, migratory, and activation states, and their relatedness within and across age. Using the cell-type-specific expression patterns of genes mutated in neurological and psychiatric diseases, we identify putative disease subtypes that associate with clinical phenotypes. Our study reveals the cellular template of a complex neurodevelopmental process, and provides a window into the cellular origins of brain diseases.

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ARTICLE

https://doi.org/10.1038/s41586-019-1506-7

# Conserved cell types with divergent features in human versus mouse cortex

Rebecca D. Hodge[1,13], Trygve E. Bakken[1,13], Jeremy A. Miller[1], Kimberly A. Smith[1], Eliza R. Barkan[1], Lucas T. Graybuck[1], Jennie L. Close[1], Brian Long[1], Nelson Johansen[2], Osnat Penn[1], Zizhen Yao[1], Jeroen Eggermont[3], Thomas Höllt[3,4], Boaz P. Levi[1], Soraya I. Shehata[1], Brian Aevermann[5], Allison Beller[6], Darren Bertagnolli[1], Krissy Brouner[1], Tamara Casper[1], Charles Cobbs[7], Rachel Dalley[1], Nick Dee[1], Song-Lin Ding[1], Richard G. Ellenbogen[8], Olivia Fong[1], Emma Garren[1], Jeff Goldy[1], Ryder P. Gwinn[9], Daniel Hirschstein[1], C. Dirk Keene[6], Mohamed Keshk[5], Andrew L. Ko[8,10], Kanan Lathia[1], Ahmed Mahfouz[3,4], Zoe Maltzer[1], Medea McGraw[1], Thuc Nghi Nguyen[1], Julie Nyhus[1], Jeffrey G. Ojemann[8,10], Aaron Oldre[1], Sheana Parry[1], Shannon Reynolds[1], Christine Rimorin[1], Nadiya V. Shapovalova[1], Saroja Somasundaram[1], Aaron Szafer[1], Elliot R. Thomsen[1], Michael Tieu[1], Gerald Quon[2], Richard H. Scheuermann[5,11], Rafael Yuste[12], Susan M. Sunkin[1], Boudewijn Lelieveldt[3,4], David Feng[1], Lydia Ng[1], Amy Bernard[1], Michael Hawrylycz[1], John W. Phillips[1], Bosiljka Tasic[1], Hongkui Zeng[1], Allan R. Jones[1], Christof Koch[1] & Ed S. Lein[1]*

**Elucidating the cellular architecture of the human cerebral cortex is central to understanding our cognitive abilities and susceptibility to disease. Here we used single-nucleus RNA-sequencing analysis to perform a comprehensive study of cell types in the middle temporal gyrus of human cortex. We identified a highly diverse set of excitatory and inhibitory neuron types that are mostly sparse, with excitatory types being less layer-restricted than expected. Comparison to similar mouse cortex single-cell RNA-sequencing datasets revealed a surprisingly well-conserved cellular architecture that enables matching of homologous types and predictions of properties of human cell types. Despite this general conservation, we also found extensive differences between homologous human and mouse cell types, including marked alterations in proportions, laminar distributions, gene expression and morphology. These species-specific features emphasize the importance of directly studying human brain.**

The cerebral cortex is responsible for our higher cognitive abilities and is the most complex structure known to biology: it comprises 16 billion neurons and 61 billion non-neuronal cells organized into more than 100 distinct anatomical or functional regions[1,2]. Human cortex is expanded relative to mouse—the dominant model organism used in research—with a more-than-1,000-fold larger area and number of neurons[3]. Whereas the general principles of cortical development and basic architecture of the cortex appear to be conserved across mammals[4], previous studies suggest differences in the cellular makeup of human cortex[5–11]. For example, superficial cortical layers are expanded in mammalian evolution[12] and some cell types, such as interlaminar astrocytes[13] and rosehip neurons[14], have specialized features in human compared to mouse. Likewise, transcriptional regulation varies between mouse and human, including the transcription of genes that are associated with neuronal structure and function[15–17].

Single-cell transcriptomics enables molecular classification of cell types, provides a metric for comparative analyses, and is fuelling efforts to understand the complete cellular makeup of the mouse brain[18] and even the entire human body[19]. Single-cell RNA sequencing (scRNA-seq) of mouse cortex demonstrates robust transcriptional signatures of cell types[20–22] and suggests around 100 cell types per cortical area. Dissociating live cells from human brain is difficult, which makes scRNA-seq challenging to apply to this type of tissue, whereas single-nucleus RNA-seq (snRNA-seq) enables transcriptional profiling of nuclei from frozen human brain specimens[23,24]. Of note, nuclei contain sufficient gene-expression information to distinguish closely related cell types at a similar resolution to scRNA-seq[25,26], but early applications of snRNA-seq to human cortex did not have sufficient depth of coverage to achieve similar resolution to mouse studies[27,28]. Here, we established robust methods for the classification of cell types in human brain using snRNA-seq and compared cortical cell types to reveal conserved and divergent features of human and mouse cerebral cortex.

## Transcriptomic taxonomy of cell types

To transcriptomically define cell types in human cortex, we used snRNA-seq and focused on middle temporal gyrus (MTG) largely from postmortem brain. MTG is often available from epilepsy resections, permitting comparison of postmortem versus live neurosurgical tissues, and enabling future correlation with in vitro slice physiology. Tissues were processed as described[14] (Fig. 1a, Extended Data Fig. 1a). Nuclei were collected from eight donor brains (Extended Data Table 1), with most coming from postmortem donors ($n = 15{,}206$) and a minority ($n = 722$) from layer (L)5 of MTG removed during neurosurgeries (Extended Data Fig. 2).

In total, 15,928 nuclei passed quality control, including those from 10,708 excitatory neurons, 4,297 inhibitory neurons and 923 non-neuronal cells. Nuclei from each broad class were iteratively clustered as described[26] (see Methods). Clusters were generally robust to different iterative clustering methods and were distinguished from nearest neighbours by at least 30 differentially expressed genes and at least one, and often more, binary markers. Requiring more binary markers led

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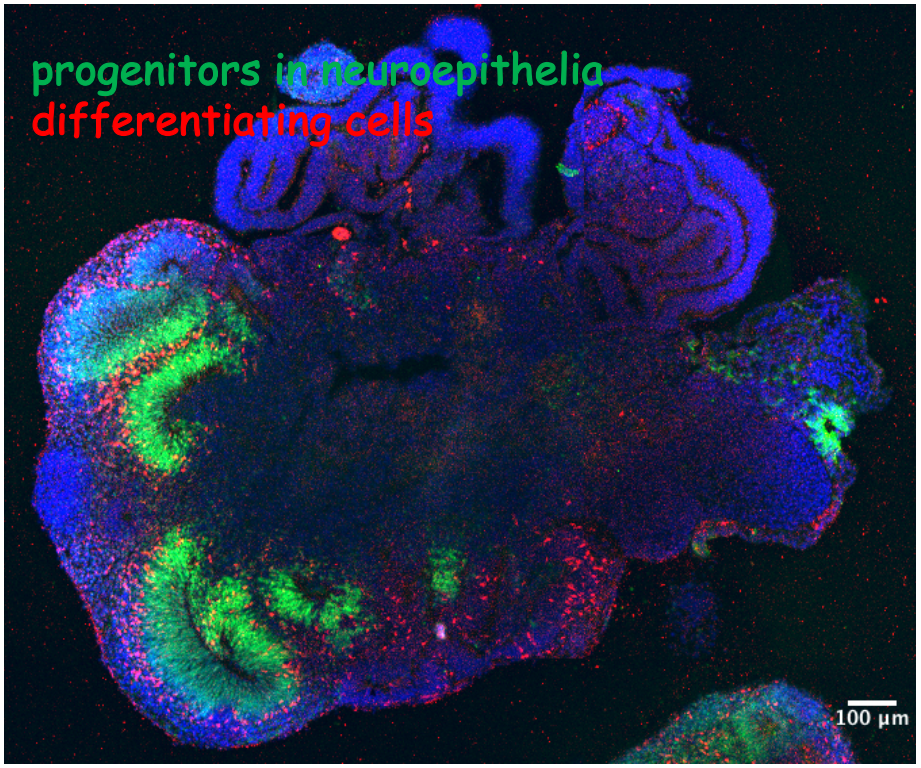
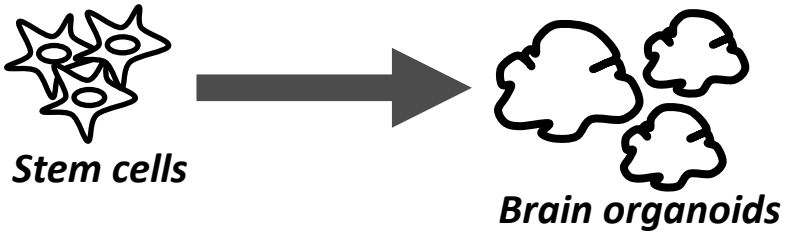
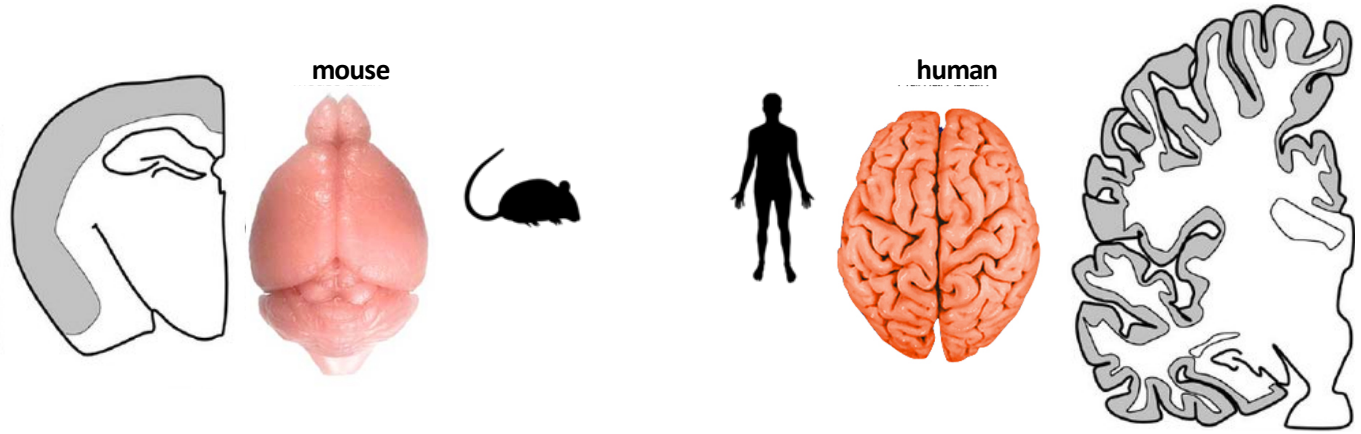
# *Human-induced pluripotent stem cells (iPSCS) & brain organoids*

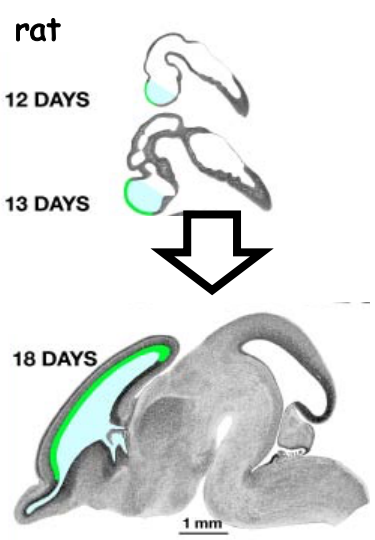
*In vitro* technique: self-assembled three-dimensional aggregates generated from pluripotent stem cells that recapitulate embryonic human brain development *(in normal or pathological conditions)*



# *Limitations of the mouse model to study neural progenitors and cortical malformations*



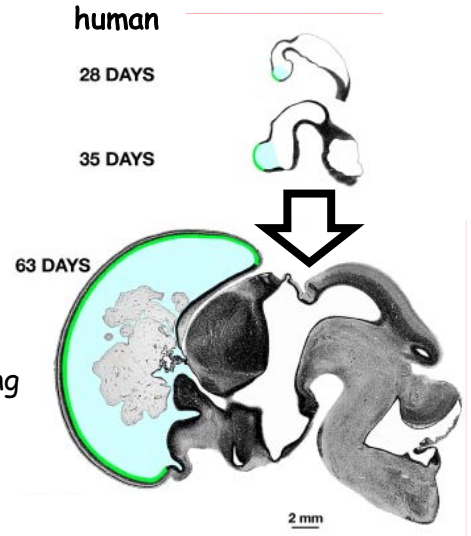




In green: future cortex

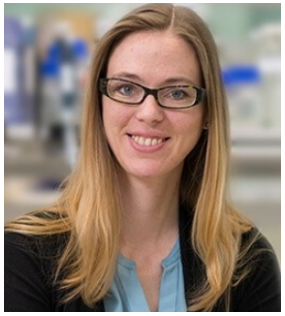
**Human corticogenesis**:

- Multiple rounds of symmetric divisions (NP pool amplification)
- Increased progenitor populations (extra layer of basal progenitors)

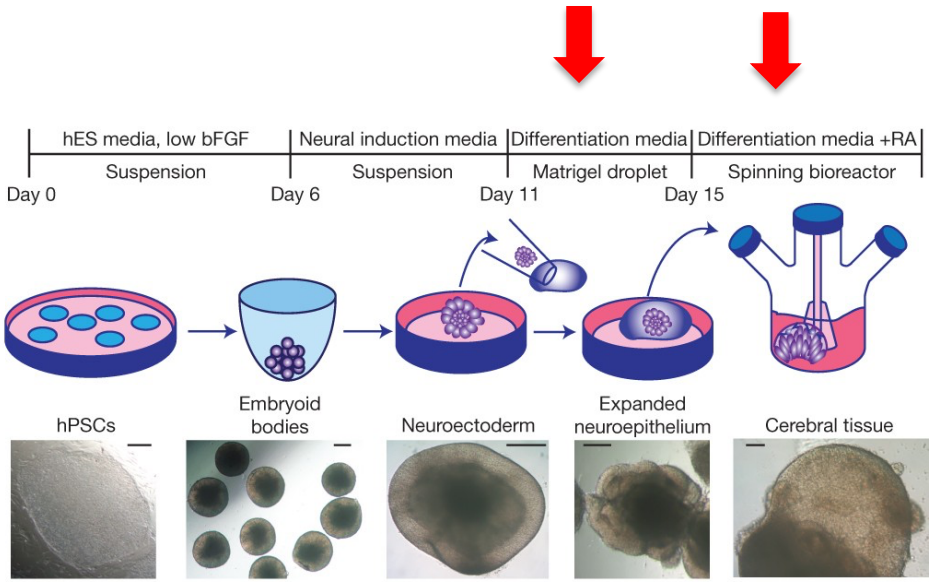
Result: Higher neuronal production and folding

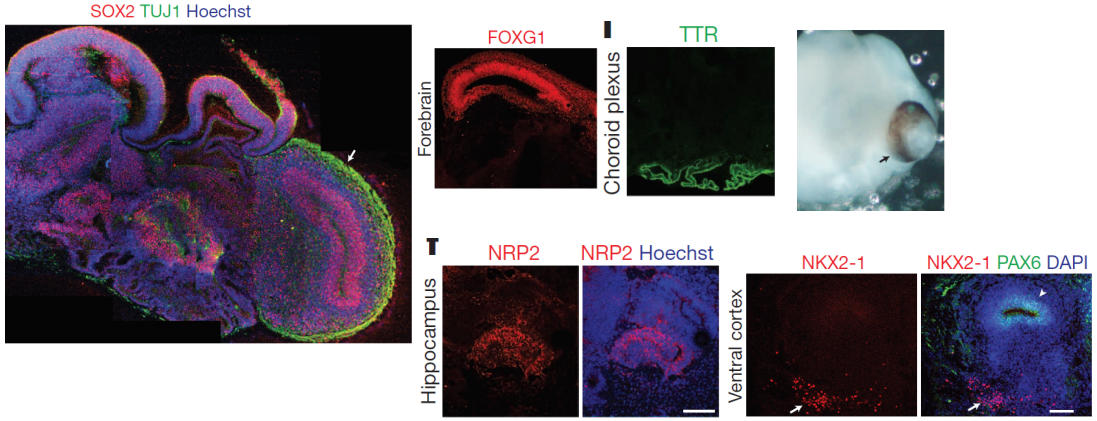


# First protocol of self-organized brain organoids



**2013**
*Various brain regions*
*Lancaster et al.*

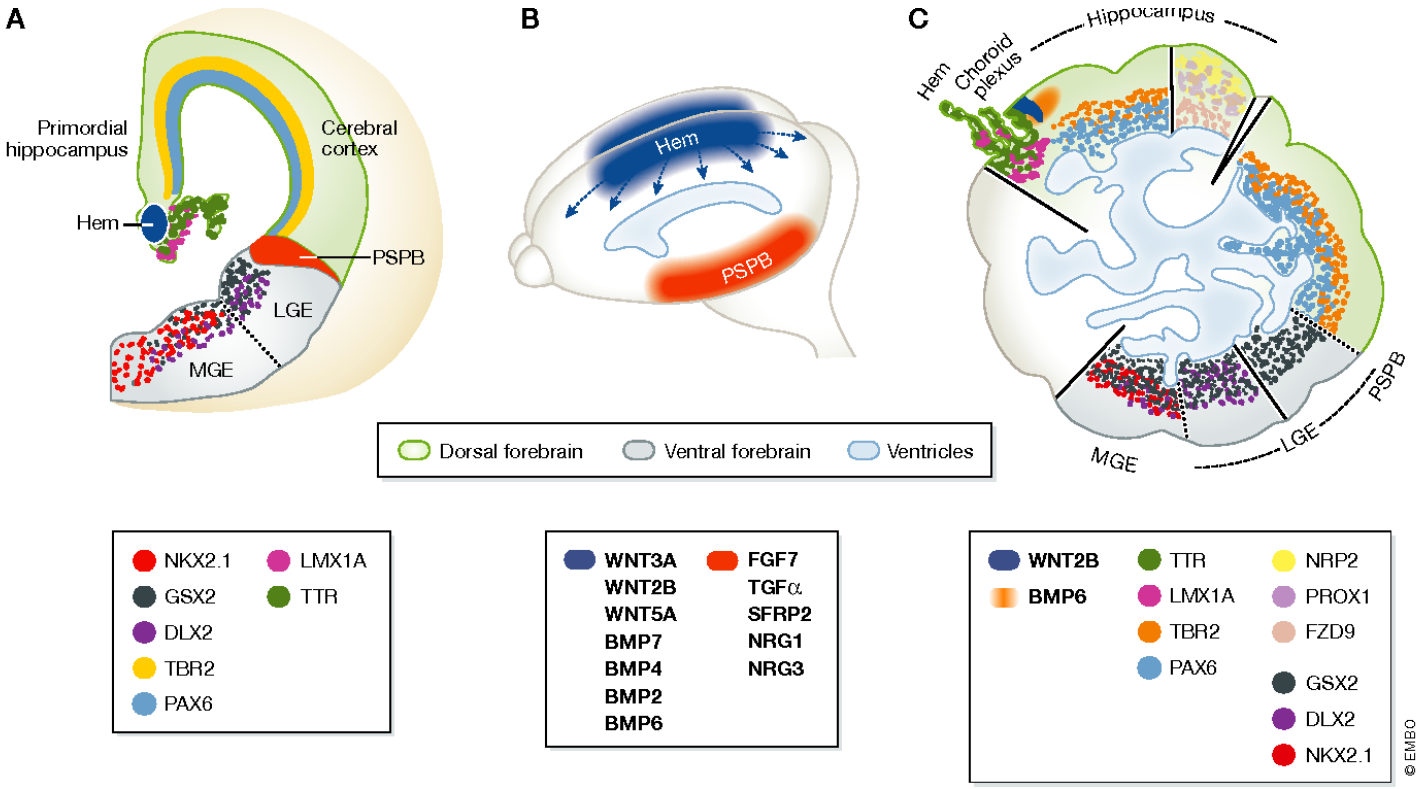



***Spontaneous differentiation (intrinsic program of self-organization) , but highly heterogenous***

*Lancaster M. et al., Nature, 2013*

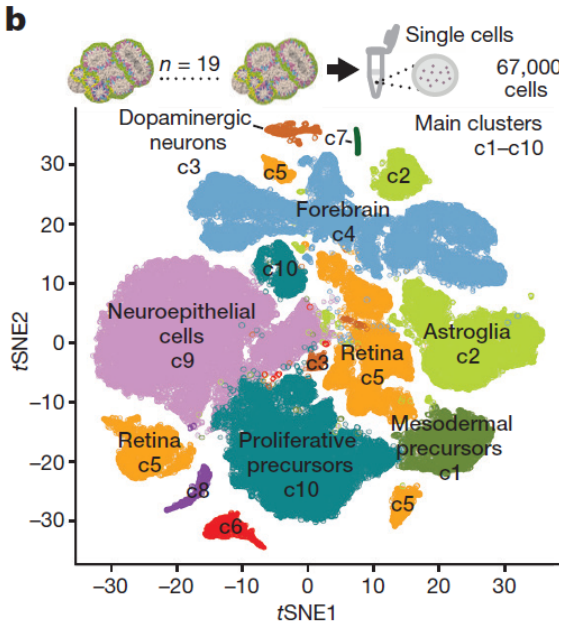
# *Self-organized developmental regional patterning and differentiation is recapitulated in cerebral organoids*




*Dias & Guillemot, EMBO J. 2017*

# *Further characterization and improvements of brain organoids*

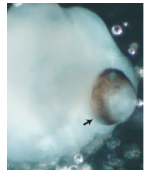
- ***Characterize***
- *Implement*
- *Simplify/Improve*
- *Patterning control*

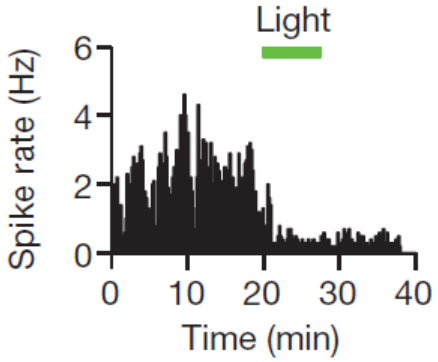



***scRNA-Seq to study heterogeneity and compare with in vivo brain cells (Lancaster, Knoblich Lab +Others)***

***2015***
***First scRNA-Seq***
*Camp et al.*

***2017***
***Photosensitive***
*organoids*
*Quadrato et al.*





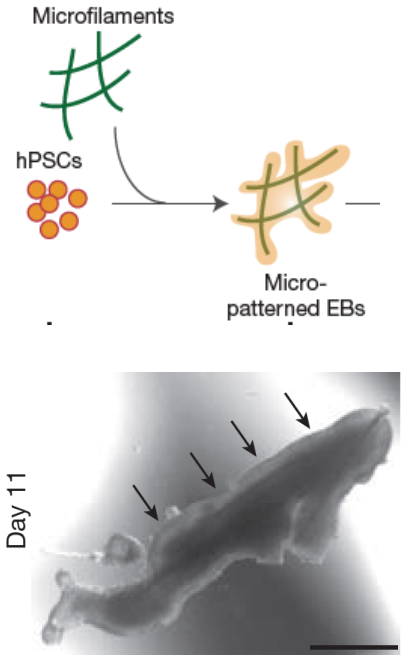

***Photosensitive retinal cells in human brain organoids (Arlotta's Lab)***

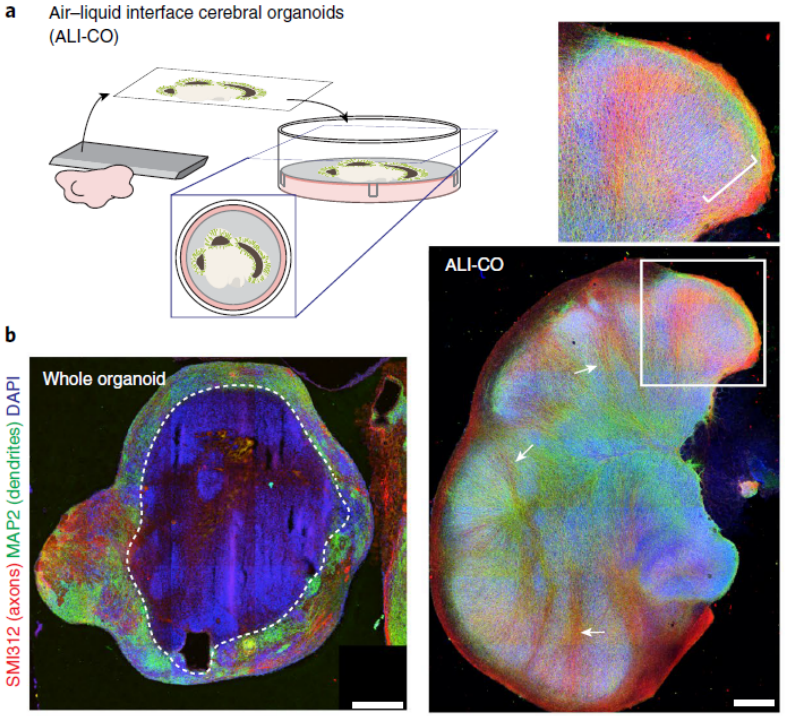
# Further characterization and improvements of brain organoids

- *Characterize*
- ***Implement***
- *Simplify/Improve*
- *Patterning control*

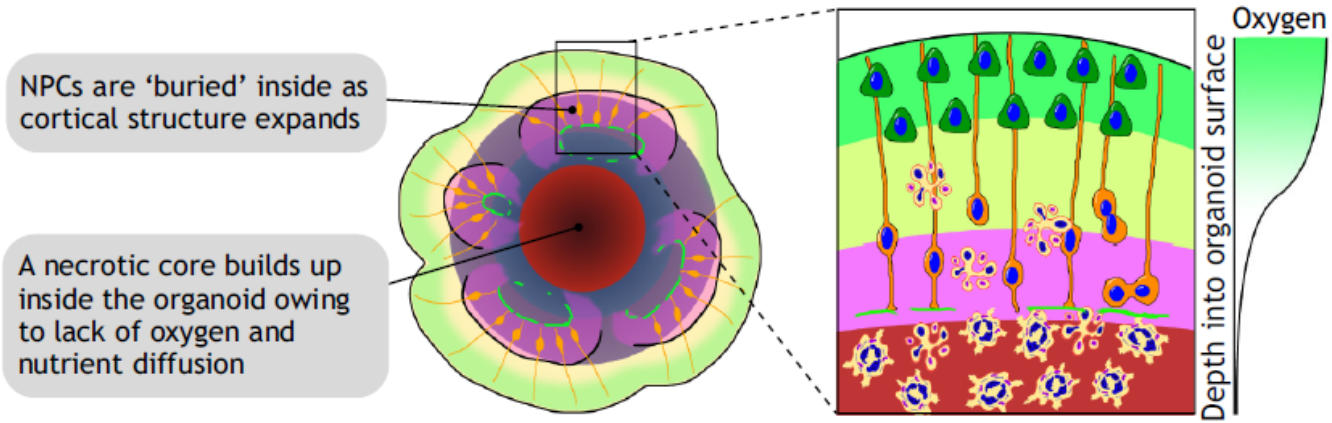
***Lancaster's Lab***
***Implementing survival and culture conditions***
***…while maintaining spontaneous differentiation***

| **2017** | **2019** |
|---|---|
| ***Biomaterials*** | ***Slicing*** |
| *Lancaster et al.* | *Giandomenico et al.* |



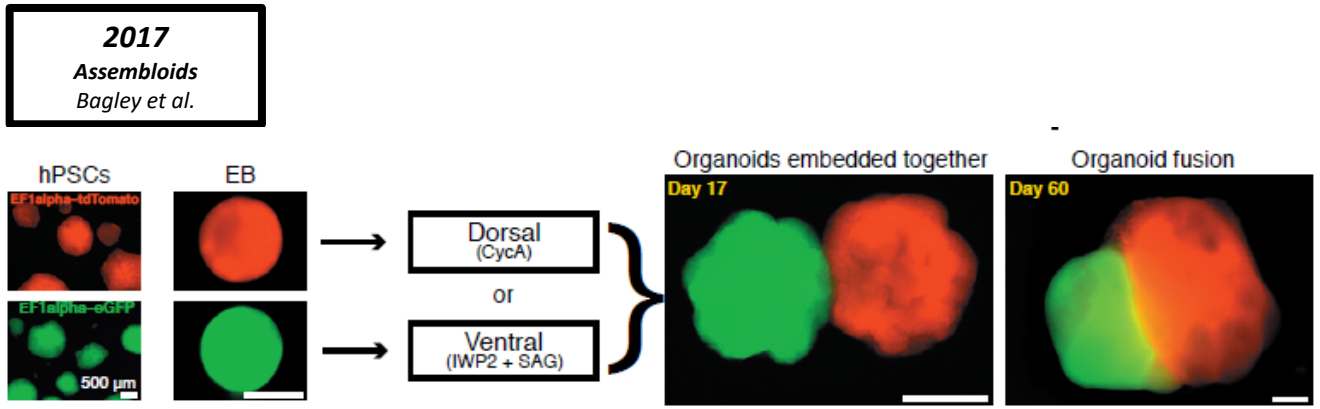
*Lack of vascularization*
*Protocol optimization: long-term survival*



# *Further characterization and improvements of brain organoids*

***Knoblich's Lab***
***Patterning is coming back: "Assembloids"***

- *Characterize*
- ***Implement***
- ***Simplify/Improve***
- ***Patterning control***

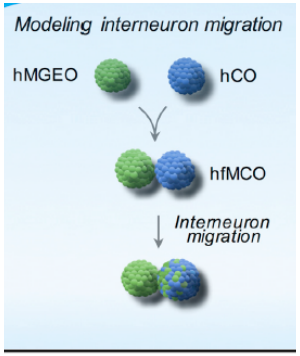


# *Further characterization and improvements of brain organoids*

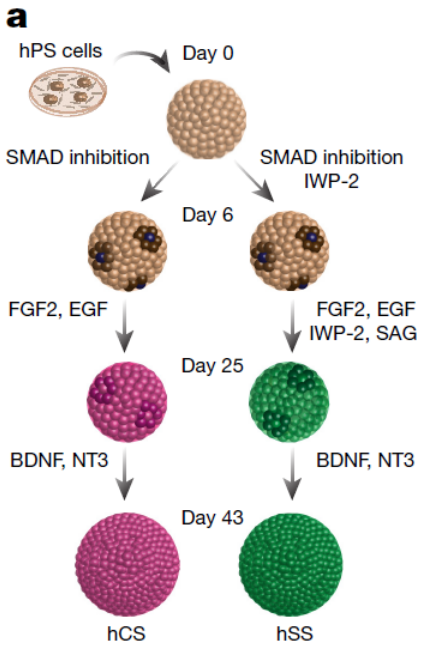
- *Characterize*
- *Implement*
- *Simplify/Improve*
- *Patterning control*

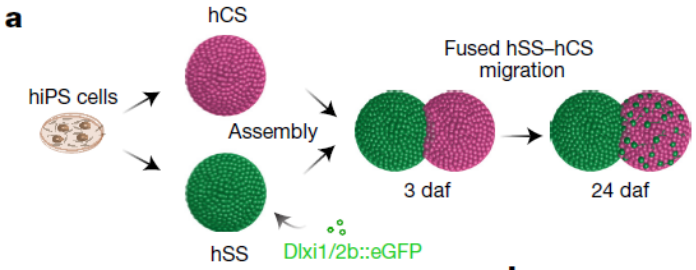
***Knoblich's Lab***
***Patterning is coming back: "Assembloids"***

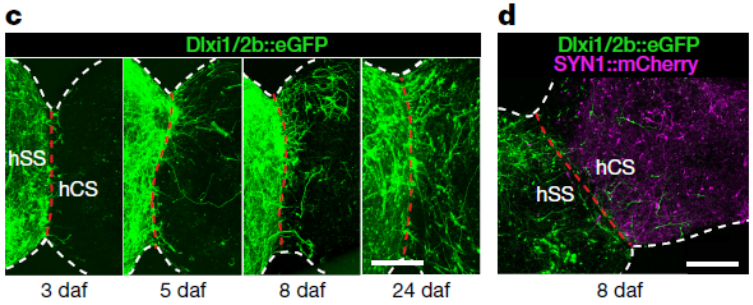
***Pasça's Lab and Park's Lab***
***No embedding in Matrigel: "Spheroids"***




**2015/2017**
***Cortical spheroids***
***Assembled spheroids***
*Pasça et al.*
*Birey et al.*
*Xiang et al.*



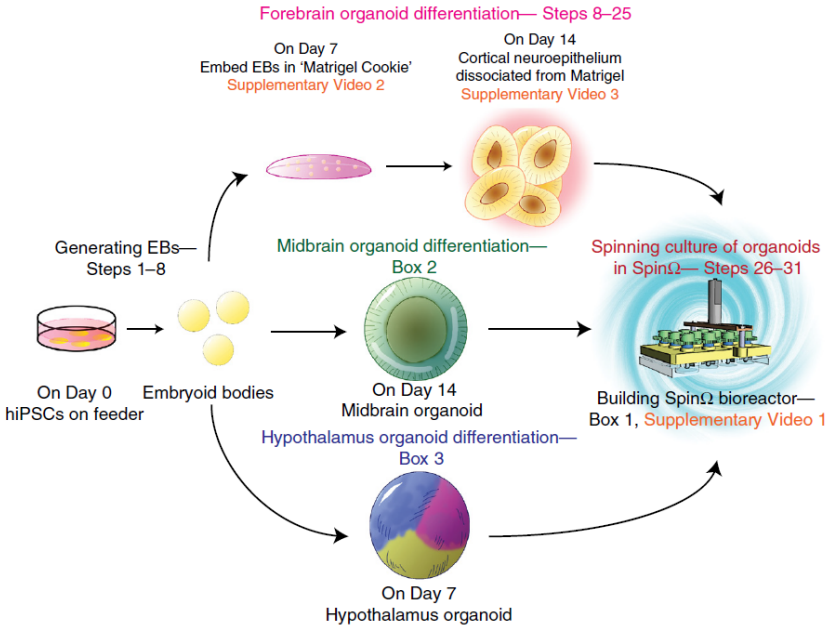



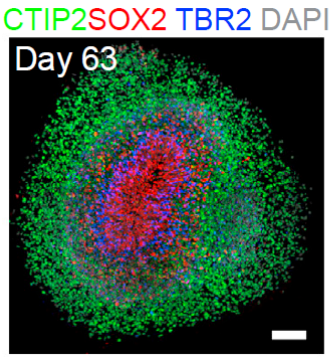
# *Further characterization and improvements of brain organoids*

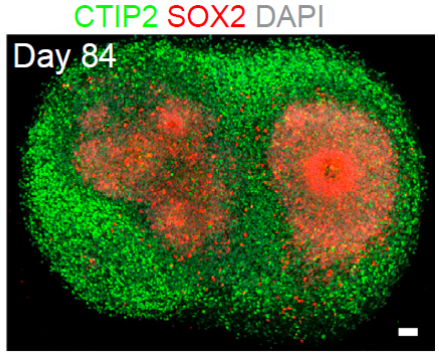
- *Characterize*
- *Implement*
- ***Simplify/Improve***
- ***Patterning control***

***Ming's Lab***
***Re-discovering patterning molecules***
***+ "Cheap" bioreactor.***
***Forebrain-, Midbrain- or***
***Hypothalamic- specific organoids***

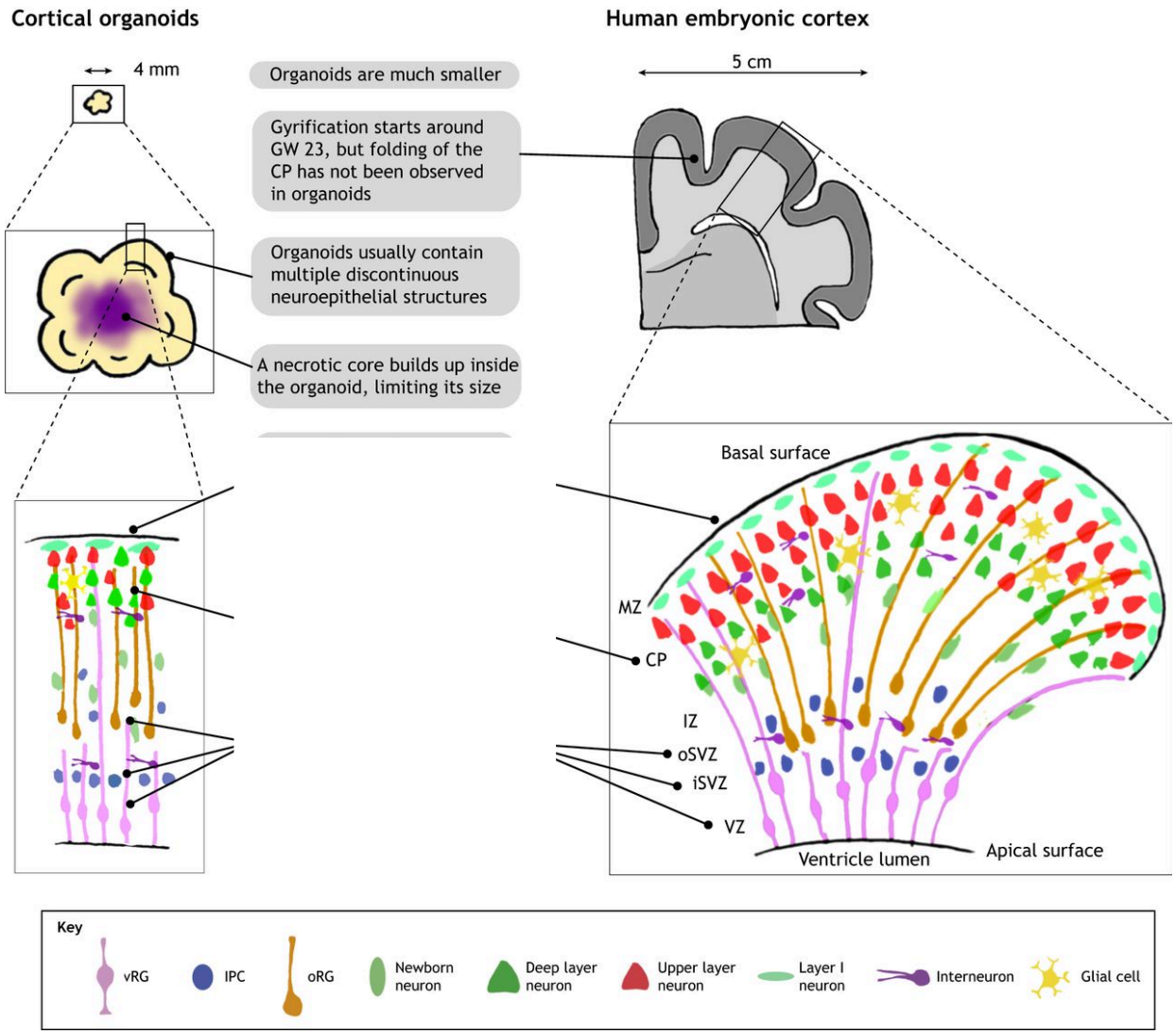



**2016-2018**
***3D-printed***
***Bioreactor***
*Qian et al.*



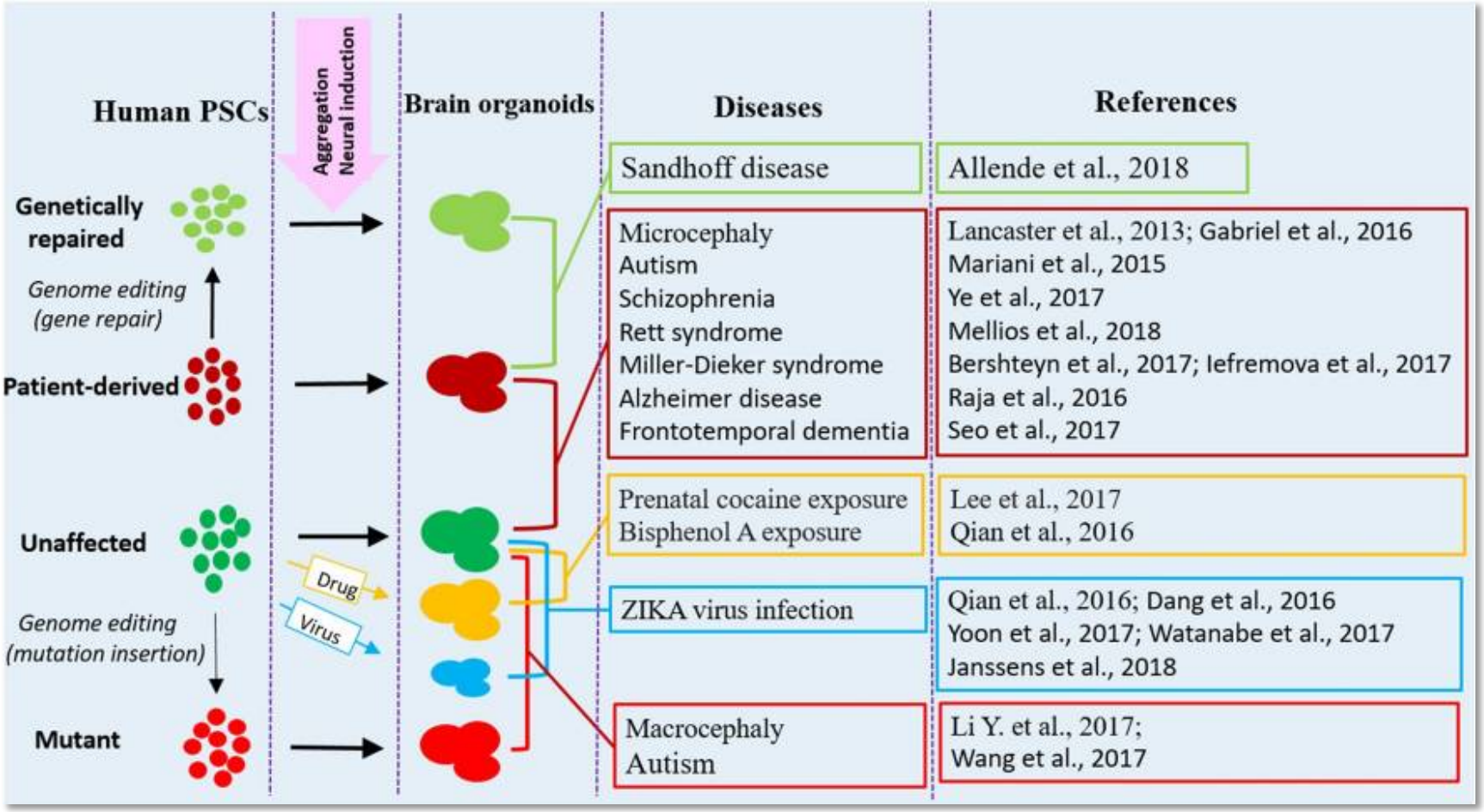



# *Structural comparison between cortical organoids and the human embryonic cortex*




Qian et al., DEV 2019

# *Organoids for human disease modeling*

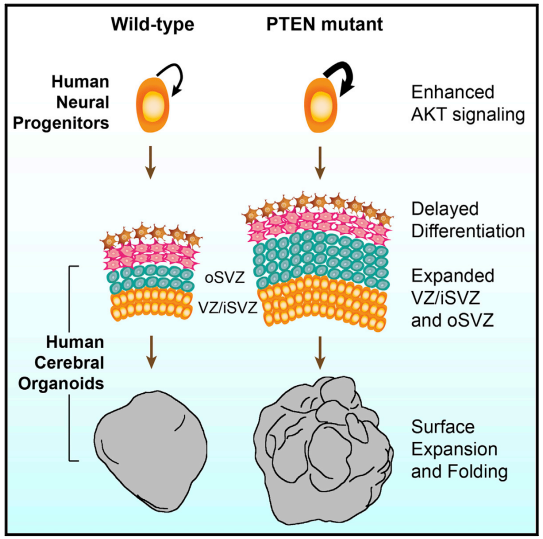



*Wang, 2018*

Cell Stem Cell

# Induction of Expansion and Folding in Human Cerebral Organoids

## Graphical Abstract





## Authors

Yun Li, Julien Muffat, Attya Omer, ..., Lee Gehrke, Juergen A. Knoblich, Rudolf Jaenisch

## Correspondence

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## In Brief

The growth and structural formation of the human cortex can be modeled in vitro using cerebral organoids. In this article, Li, Muffat, and colleagues show that enhancing the PTEN-AKT signaling pathway leads to the generation of human cerebral organoids that are expanded in size and display surface folding.


## Highlights

- PTEN deletion enhances human NP proliferation and sensitivity to growth factors
- PTEN mutant human, but not mouse, cerebral organoids display surface folding
- Folded human organoids contain expanded ventricular and outer progenitors
- Zika virus impairs the formation of expanded and folded human cerebral organoids

Li et al., 2017, Cell Stem Cell *20*, 385–396
March 2, 2017 
http://dx.doi.org/10.1016/j.stem.2016.11.017

CellPress

nature medicine

LETTERS

https://doi.org/10.1038/s41591-019-0371-0

# Altered neuronal migratory trajectories in human cerebral organoids derived from individuals with neuronal heterotopia

Johannes Klaus[1,11], Sabina Kanton[2,11], Christina Kyrousi[1,11], Ane Cristina Ayo-Martin[1,3], Rossella Di Giaimo[1,4], Stephan Riesenberg[2], Adam C. O'Neill[5,6], J. Gray Camp[2], Chiara Tocco[1], Malgorzata Santel[2], Ejona Rusha[7], Micha Drukker[7], Mariana Schroeder[1], Magdalena Götz[6,8], Stephen P. Robertson[5], Barbara Treutlein[2,9,10]* and Silvia Cappello[1]*

**Malformations of the human cortex represent a major cause of disability[1]. Mouse models with mutations in known causal genes only partially recapitulate the phenotypes and are therefore not unlimitedly suited for understanding the molecular and cellular mechanisms responsible for these conditions[2]. Here we study periventricular heterotopia (PH) by analyzing cerebral organoids derived from induced pluripotent stem cells (iPSCs) of patients with mutations in the cadherin receptor–ligand pair *DCHS1* and *FAT4* or from isogenic knockout (KO) lines[1,3]. Our results show that human cerebral organoids reproduce the cortical heterotopia associated with PH. Mutations in *DCHS1* and *FAT4* or knockdown of their expression causes changes in the morphology of neural progenitor cells and result in defective neuronal migration dynamics only in a subset of neurons. Single-cell RNA-sequencing (scRNA-seq) data reveal a subpopulation of mutant neurons with dysregulated genes involved in axon guidance, neuronal migration and patterning. We suggest that defective neural progenitor cell (NPC) morphology and an altered navigation system in a subset of neurons underlie this form of PH.**

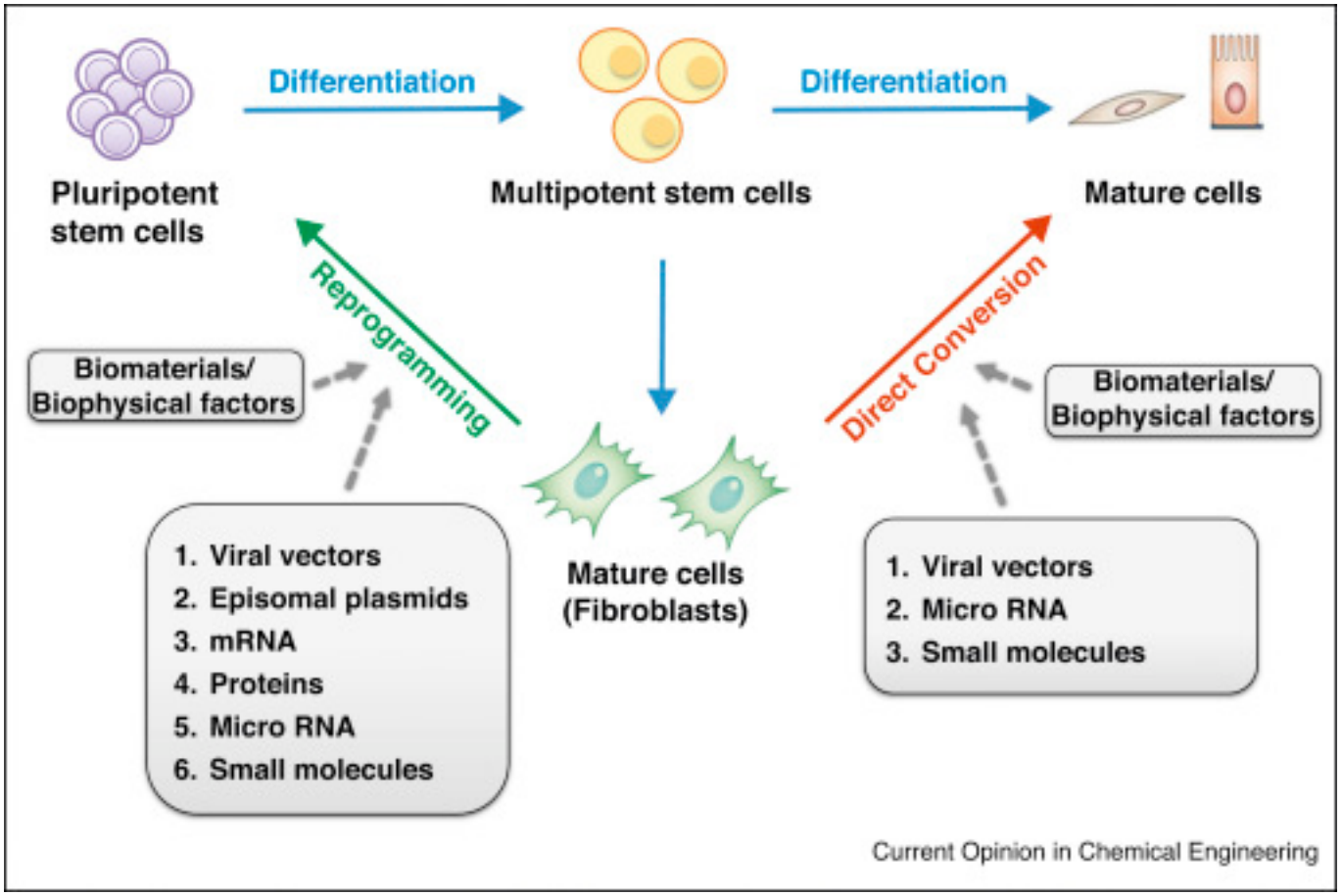
Mammalian neocortical development represents a highly orchestrated process that depends on the precise generation, migration and maturation of neurons. The importance of a coordinated sequence is underlined by the conditions with its disruption: malformation of cortical development. PH represents one of the most common forms of these disorders and is characterized by heterotopic neurons lining their sites of production. Patients with PH typically present with intellectual disability, and this is frequently associated with epilepsy[4–10]. The identification of mutations in the protocadherins *DCHS1* and *FAT4* put the spotlight on defects in NPCs as a causal mechanism of the condition. Here we explore the functions of DCHS1 and FAT4 in the developing cortex using human iPSC-derived NPCs, neurons and cerebral organoids.

We first reprogrammed fibroblasts from control individuals and patients with PH who carry mutations in *DCHS1* or *FAT4* into iPSCs (Extended Data 1a,b). Specifically, fibroblasts were collected from two different previously characterized patients[1,3]: one was compound heterozygous for mutations in the *FAT4* gene and one homozygous for mutation in the *DCHS1* gene. Additionally, to control for differences due to the different genomic background in the patients, we generated KO iPSC lines for both genes using CRISPR–Cas9 genome editing in control iPSCs. We programmed the iPSCs toward NPCs and neurons in two-dimensional (2D) culture and generated three-dimensional (3D) cerebral organoids[11] (Extended Data 1c,d). Using in situ hybridization, we identified that both genes were expressed in the periventricular structures of cerebral organoids and neurons (Extended Data 1e–h′), a pattern consistent with that detected in mouse and human[1,12]. These findings were confirmed by scRNA-seq of cells derived from cerebral organoids, where the expression of *DCHS1* and *FAT4* was found in both progenitors and neurons (Extended Data 1i,j).

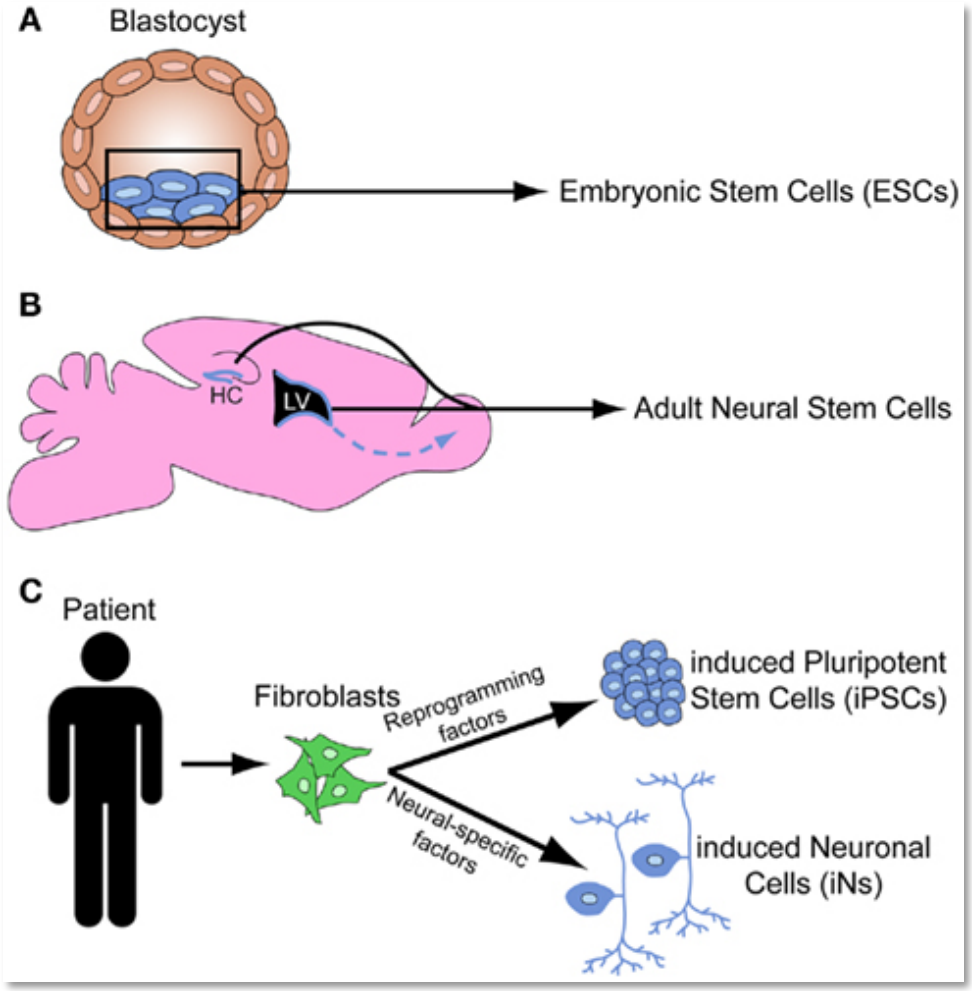
To investigate whether PH is recapitulated within cerebral organoids (Extended Data 1k,l), we scrutinized ventricular zone structures for such phenotypes. In control organoids, a clear distinction of the neuronal (MAP2+ cells or NEUN+ cells) layer from the germinal zone (PAX6+ cells) was identified (Fig. 1a,d, Extended Data 2a,e,h and Extended Data 3g,k). Organoids derived from mutant or KO iPSC lines exhibited a significant number of neuronal nodules at ventricular positions (Fig. 1a–c, Extended Data 2e–j, and Extended Data 2l–l‴). In addition to this neuronal heterotopia-like phenotype, mutant and KO organoids presented poorly organized germinal zones (Fig. 1d,e and Extended Data 2d). This feature was especially apparent in germinal zones of *FAT4*-mutant organoids, with most not showing evident separation of the neuronal band from the germinal zone, with neurons intruding in most cases. In the case of *DCHS1*-mutant or knockdown organoids, performed via electroporation of specifically designed microRNAs (miRNAs) that target the human gene (Extended Data 2k), clusters of neurons were found within the germinal zones where NPC processes where disrupted (Fig. 1f–f‴ and Extended Data 2l–l‴). Although *DCHS1*-mutant organoids displayed clearer separations between the germinal zones and neuronal layer, the neurites showed an

[1]Max Planck Institute of Psychiatry, Munich, Germany. [2]Max Planck Institute for Evolutionary Anthropology, Leipzig, Germany. [3]International Max Planck Research School for Translational Psychiatry (IMPRS-TP), Munich, Germany. [4]Department of Biology, University of Naples Federico II, Naples, Italy. [5]Department of Women's and Children's Health, University of Otago, Dunedin, New Zealand. [6]Institute of Stem Cell Research, Helmholtz Center Munich, Munich, Germany. [7]Institute of Stem Cell Research, iPSC Core Facility, Helmholtz Center Munich, Munich, Germany. [8]Ludwig Maximilian University, Munich, Germany. [9]Max Planck Institute of Molecular Cell Biology and Genetics, Dresden, Germany. [10]Technical University Munich, Department of Biosciences, Freising, Germany. [11]These authors contributed equally: Johannes Klaus, Sabina Kanton, Christina Kyrousi. *e-mail: barbara_treutlein@eva.mpg.de; silvia_cappello@psych.mpg.de

# 5. In vitro & In vivo reprogramming



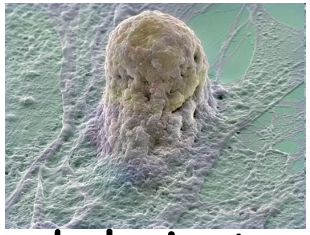
# *Generating cortical neurons in vitro to re-implant in vivo*



# *Indirect and direct lineage reprogramming to create patient-derived neural cells*

patient skin samples

Reprogramming genes

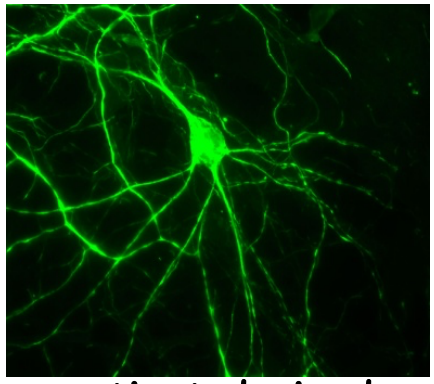


Induced pluripotent stem cells (iPSC)

Differentiate

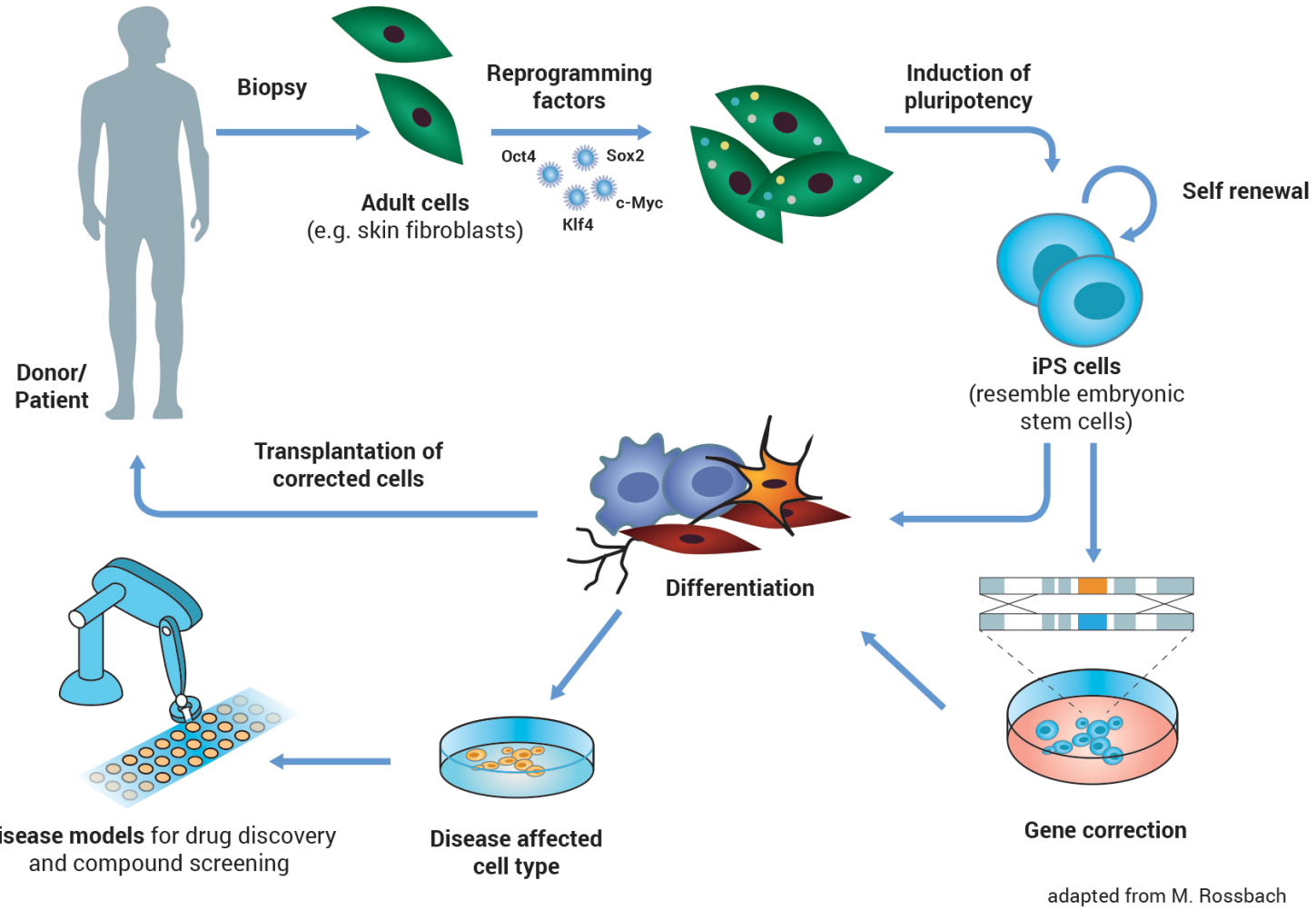
Direct conversion

Induced Neuronal (iN) cells



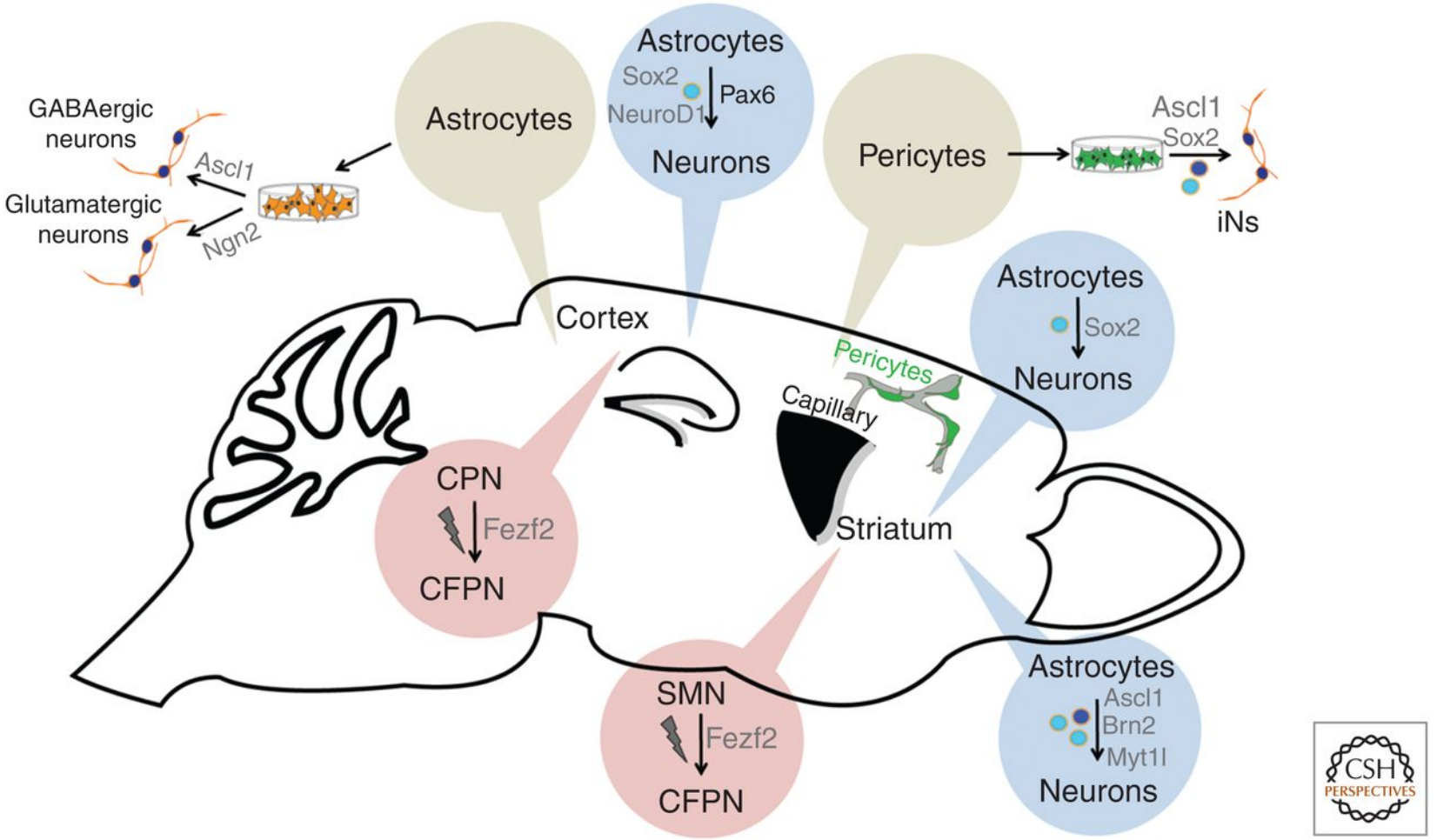
patient-derived neurons

# Regenerative medicine in clinics and industry



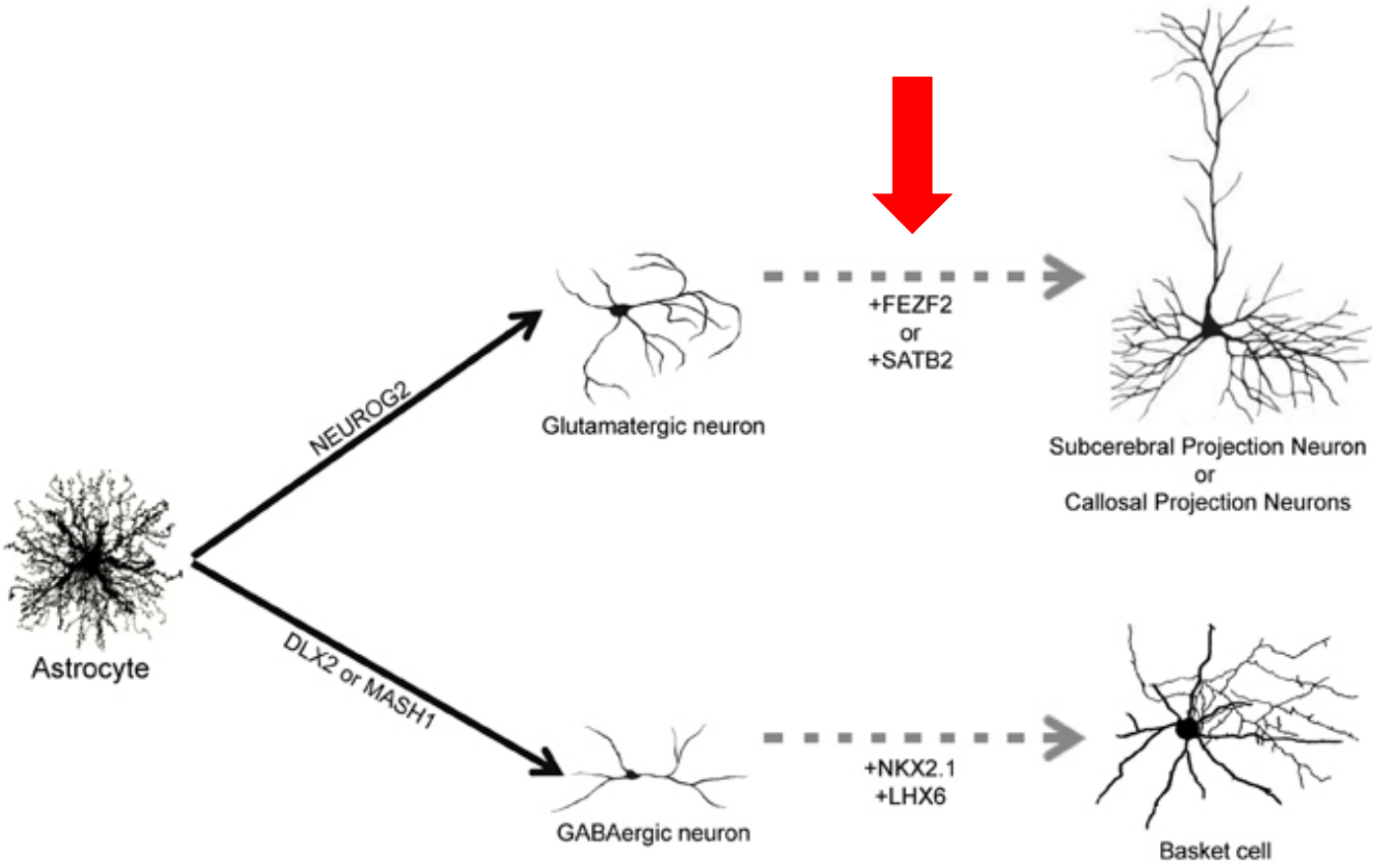

adapted from M. Rossbach

# Engineering neurogenesis outside the classical neurogenic niches




Berninger and Jessberger, 2016

# *Somatic cell direct reprogramming in the brain*



ARTICLES
https://doi.org/10.1038/s41593-018-0168-3
nature neuroscience

# Direct pericyte-to-neuron reprogramming via unfolding of a neural stem cell-like program

Marisa Karow[1,2,14*], J. Gray Camp[3,14], Sven Falk[2,4], Tobias Gerber[3], Abhijeet Pataskar[5], Malgorzata Gac-Santel[3], Jorge Kageyama[3], Agnieska Brazovskaja[3], Angela Garding[5], Wenqiang Fan[1], Therese Riedemann[2], Antonella Casamassa[1,13], Andrej Smiyakin[6], Christian Schichor[7], Magdalena Götz[2,4], Vijay K. Tiwari[5], Barbara Treutlein[3,8,9,15*] and Benedikt Berninger[1,10,11,12,15*]

**Ectopic expression of defined transcription factors can force direct cell-fate conversion from one lineage to another in the absence of cell division. Several transcription factor cocktails have enabled successful reprogramming of various somatic cell types into induced neurons (iNs) of distinct neurotransmitter phenotype. However, the nature of the intermediate states that drive the reprogramming trajectory toward distinct iN types is largely unknown. Here we show that successful direct reprogramming of adult human brain pericytes into functional iNs by Ascl1 and Sox2 encompasses transient activation of a neural stem cell-like gene expression program that precedes bifurcation into distinct neuronal lineages. During this transient state, key signaling components relevant for neural induction and neural stem cell maintenance are regulated by and functionally contribute to iN reprogramming and maturation. Thus, Ascl1- and Sox2-mediated reprogramming into a broad spectrum of iN types involves the unfolding of a developmental program via neural stem cell-like intermediates.**

Direct lineage reprogramming is an emerging strategy for harnessing the cellular plasticity of differentiated cells for lineage conversion into desired target cell types for disease modeling and tissue repair[1–4]. While direct lineage reprogramming from starting to target-cell type classically occurs without cell division, thereby sharply contrasting with reprogramming toward induced pluripotency[5], little is known about the intermediate states that bridge the trajectory between start and end points. Two models have been proposed, according to which direct reprogramming is mediated either through direct conversion between fully differentiated states or through reversal to a developmentally immature state[6]. Furthermore, reprogramming efficiency and final differentiation outcomes are highly cellular-context-dependent, for which the underlying reasons are only incompletely understood[7,8]. Analyses of the transcriptome alterations induced by the reprogramming factors have yielded fundamental insights into the molecular mechanisms of iN conversion[9–12]. For instance, a single factor, Ascl1, can reprogram mouse astrocytes into iNs with high efficiency[13], while the same factor induces a muscle cell-like fate in mouse embryonic fibroblasts (MEF) alongside neuronal fates[11,14]. Efficient reprogramming of MEFs into iNs requires co-expression of additional factors (for example, Brn2, Ascl1 and Myt1l, collectively referred to as BAM)[9,11,12,15]. Moreover, Ascl1 induces a GABAergic neuron identity in mouse astrocytes[10,13], while BAM-transduced fibroblasts predominantly adopt a glutamatergic phenotype[15], raising questions of how the respective reprogramming trajectories translate into distinct iN transmitter and subtype identities.

In the present study, by analyzing transcriptomes at population and single cell level, we aimed to reconstruct the trajectories underlying direct lineage conversion of adult human brain pericytes into iNs by forced expression of Ascl1 and Sox2 (AS)[16]. This allowed us to scrutinize the contribution of the starting cell population's heterogeneity to the variability in reprogramming success. By identifying cells of distinct reprogramming competence, we were able to reconstruct a trajectory of productive AS-mediated iN generation, allowing us to uncover intermediate states during successful conversion. Unexpectedly, we found that despite the absence of cell division, cells in the productive trajectory passed through a neural stem cell-like state. Transiently induced genes, many of which are core components of signaling pathways, typified this intermediate state, and interference with these signaling pathways demonstrated their functional importance for the reprogramming process. Finally, the productive reprogramming trajectory revealed an unexpected point of bifurcation into lineages whose transcriptomes were dominated by transcription factor families involved in the specification of GABAergic and glutamatergic subclasses of forebrain neurons.

## Results

**Ascl1 and Sox2 synergism in inducing neuronal gene expression in pericytes.** We have recently shown that adult human brain

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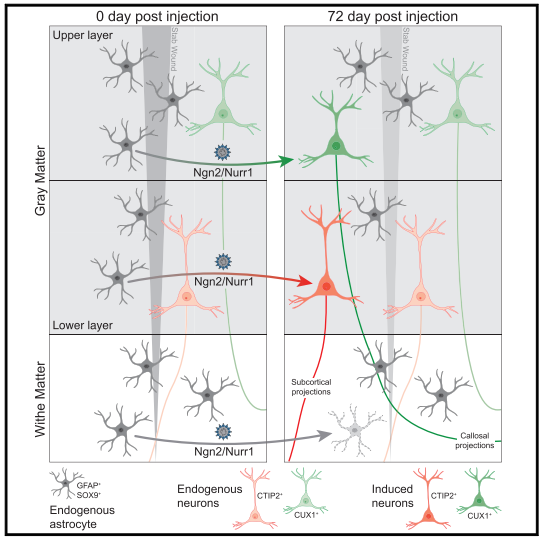
Neuron

# Inducing Different Neuronal Subtypes from Astrocytes in the Injured Mouse Cerebral Cortex

## Graphical Abstract





## Authors

Nicola Mattugini, Riccardo Bocchi, Volker Scheuss, Gianluca Luigi Russo, Olof Torper, Chu Lan Lao, Magdalena Götz

## Correspondence

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## In Brief

Neurons dying after brain injury cannot be replaced. Mattugini, Bocchi, et al. show that local astrocytes can be converted into functional neurons acquiring appropriate layer identity and connectivity by expression of neurogenic factors in a mouse model of traumatic brain injury.


## Highlights

- AAV can be targeted to reactive astrocytes upon stab wound injury
- Expression of Ngn2 and Nurr1 in these astrocytes induces pyramidal neurons
- Induced pyramidal neurons acquire correct layer identity and axonal projections
- Neurons cannot be induced in the white matter

Mattugini et al., 2019, Neuron *103*, 1086–1095
September 25, 2019 
https://doi.org/10.1016/j.neuron.2019.08.009

CellPress

# *Developmental Neurobiology - Cortical Development*
# *Week 3*

***Tuesday May 19th***

*11:00-13:00* – **Student presentation on novel technologies**
*(5 groups - 2-3 per group → 15-20' per group + questions)*