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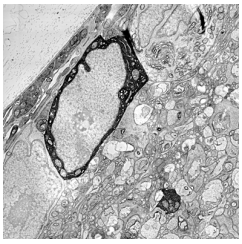
Abstract

Developments in bioengineering and molecular biology have introduced a palette of genetically encoded probes for identification of specific cell populations in electron microscopy. These probes can be targeted to distinct cellular compartments, rendering them electron dense through a subsequent chemical reaction. These electron densities strongly increase the local contrast in samples prepared for electron microscopy, allowing three major advances in ultrastructural mapping of circuits: genetic identification of circuit components, targeted imaging of regions of interest and automated analysis of the tagged circuits. Together, the gains from these advances can decrease the [illegible]nitude. These genetic encoded tags for electron microscopy (GETEM) promise to simplify the analysis of circuit motifs and become a central tool for structure-function studies of synaptic connections in the brain. We will review the current state-of-the-art with an emphasis on connectomics, the quantitative analysis of neuronal structures and motifs.

Graphical/Visual Abstract and Caption



Genetic encoded probes for electron microscopy - the toolbox for targeted ultrastructural dissections of neuronal circuit motifs.

Introduction

Our ability to sense, move, learn and interact with the world is controlled by billions of neurons interconnected via trillions of synapses. These neuronal components form complex neuronal circuits that connect distinct brain regions and build computational units in precisely defined micro-circuitries. Understanding the computational transformations and the behavioral relevance of these structures is one of the main goals of neuroscience[1,2]. This goal appears to be approachable from a physiological perspective, thanks to the palette of new genetic tools available to disrupt, modify and analyze these circuitries *in vivo* and in behaving animals[3,4]. However, to unambiguously define the mechanisms of neuronal processing, understanding the precise connectivity patterns of these circuitries is also required. These connectivity patterns, also known as 'connectomes', are comparable to the wiring diagrams an engineer would study to understand an electronic device. Similarly, biological connectomes contain a clear description of the flow of information by precisely defining the input and output components in a quantitative manner. Moreover, connectomes describe the spatial distribution of neuronal interactions, constraining the theoretically plausible mechanisms of neuronal computations.

To map neuronal circuits and generate the required wiring diagrams, several techniques have been developed in the past. Classical neuronal tracing methods with anterograde and retrograde tracers (horseradish peroxidase (HRP), wheat-germ agglutinin (WGA), *Phaseolus vulgaris* leucoagglutinin (PHAL), cholera toxin B subunit (CTB), biotinylated dextran (BDA), etc.) have provided an extensive diagram of neuronal projections but usually lack cell-type specificity and quantitative measures of synaptic connections, and remain limited in dissecting local microcircuits[5]. Virus-mediated tracing methods utilize promotor/enhancer sequences to express various probes in specific cell types[6], but identification of synaptic connections is not feasible with light microscopic examination. Some innovative light microscopic methods such as GRASP[7], super-resolution imaging[8] and trans-synaptic viral tracing[9] enable the validation of synaptic connection in identified neurons. Electron microscopy (EM), however, is the only current technique available that provides the needed resolution to visualize all essential components of a neuronal circuit, including number of synapses of different types and morphometries of synaptic connections. The standard approach is to generate electron microscopic volumes by serially imaging ultrathin sections (< 30 nm) at nanometer resolution. There are several different strategies currently being used. The most established ones, known as ATUM ('automated tape-collecting ultramicrotomy')[10] and SBEM ('serial block-face electron microscopy')[11,12], use scanning electron microscopy. Other approaches based on transmission electron microscopy are also being developed[13,14], though with more laborious tasks and higher risk

of losing complete samples. Recent progress and problems of these techniques have been discussed in previous reviews dedicated to this topic[15-17].

To investigate circuit motifs and validate circuit models routinely, the acquisition and analysis of these data-sets should be efficiently accomplished in less than a "PhD's lifespan". Recent developments in imaging technologies have improved imaging acquisition speed dramatically. Multi-beam serial-scanning electron microscopy (multi-SEM) increases image acquisition speeds by > 200-fold compared to conventional single beam electron microscopes, reaching acquisition speeds of > 1 GHz[18]. This technological advance could enable, e.g., the acquisition of the complete connectome of a mouse brain at nanometer resolution in 3 years[17,19]. Multi-SEM, however, is an expensive new technology deployed only in very few institutions. Most laboratories working in the field use single beam SEM, slowing their acquisition dramatically. But the acquisition speed is not the bottleneck for most projects. The biggest time limiting factors are the segmentation and reconstruction of all neuronal processes. Although the reconstruction of all processes in small brain volumes of around 1500 μm^3 have been demonstrated to be feasible[20], it remains extremely challenging due to the needed workforce based on semiautomatic or manual annotation approaches[14,20-22], including crowdsourcing tools[23]. For example, a seminal work to map the complete cell types of the retina by "skeletonization" (the annotation of the center of a process across sections) and cross-validation of all processes in an ~114 x 80 x 80 μm^3 small EM-volume took > 20.000 annotator hours[21], clearly more than a "PhD's lifespan". Although progress has been made in automatic algorithms[24-26], automatic annotation remains unsolved[16]. If this remains so, the speed acquisition gained with the newest imaging technologies will make this problem even worse. Thus, despite the importance of neuronal wiring diagrams for neuroscience, complete connectomes are still difficult to generate and remain a niche technology.

The reconstruction of all elements in a brain, although rich in information, is not always required to answer scientific questions[14,22,27-29]. In neuroscience, most projects focus on either defined brain areas or microcircuits, compare different phenotypes, genotypes or experimental conditions. Most of these projects are currently not accessible for a connectomics analysis due to the enormous resources they would consume by comparing multiple samples. In these cases, however, a 'sparse' reconstruction of a brain area is sufficiently informative. 'Sparse' reconstruction describes the analysis of a subset of neuronal processes in a given brain area, e.g. the connectivity of cells previously imaged for Ca^{2+} signals[14,28] or the input connectivity between two defined cell types[22,29]. These approaches are now greatly simplified by genetically encoded tags for electron microscopy (GETEM) that can help to target imaging efforts to particular brain regions and neuronal populations. These methods not only reduce imaging time and efforts, but also simplify the analysis by targeting elements of genetically defined circuit motifs[30-32]. Here we argue in favor of this parallel approach that could overcome certain intrinsic difficulties of 'dense' reconstructions. We will review the existing probes available and emphasize the strength, challenges and limitations of these approaches for the analysis of neuronal circuits.

GETEM, a recollection

The function of GETEM is to render specific structures in electron micrographs clearly visible by enhancing their contrast and thus, identify defined cell types. In the past, the most common approaches to identify have been antibody-based detection methods. These either use HRP- and

gold particle-coupled antibodies for directly detect antigens in specific neuronal compartments in EM[33-35], or fluorescent labeled antibodies for a combined optical and electron microscopic approach[36]. When these methods are used in intact brain samples, permeabilizing treatments that strongly degrade the ultrastructure are required and thus, not suitable for connectomics. Genetically encoded tags do not require these permeabilization, because they are already targeted to the site of interest before fixation. The most common chemical reaction is the polymerization of 3,3'-diaminobenzidine (DAB). Polymerization is driven by an oxidizing agent or a photo-chemical reaction that is generated by GETEM. This polymer is photon-dense and clearly visible as a brownish precipitate using light microscopy. Furthermore, it can react with osmium-tetroxide. Osmium, as well as all heavy metals, scatter the imaging electrons, thus giving the structures it is bound to higher contrast in electron micrographs. The repertoire of GETEM has been growing steadily in the last years. Three different families of genetically encoded tags have emerged as useful tools for tagging cells in electron microscopy: peroxidases, phosphatases and singlet oxygen generators (Fig. 1). All of them are based on the same premise, to localize a chemical reaction that can be rendered electron dense. These tools promise to be useful to bridge the gap between structure and function. We will review these developments by family and discuss the general constraints of these tools.

Peroxidases

The best-known peroxidase used as a tracer for anatomical studies is horseradish peroxidase (HRP). Extracted from the root of the horseradish, HRP revolutionized neuroanatomical studies in the 1970s when it was applied to trace axonal nerve fibers through retrograde transport[37,38] or injected into single cells to correlate their physiological properties with anatomical features[39,40]. HRP was also the first recombinantly produced peroxidase. It was quickly noticed that the functionality of this protein was strongly influenced by the targeting site, as it is enzymatically inactive in the cytosol due to its inability to form disulfide bonds or become glycosylated. This limitation was partially overcome by directing the protein to the extracellular membrane or topologically extracellular compartments, such as endoplasmic reticulum and synaptic vesicles[32,41-44] (Fig. 1 A1-2, C), an approach that has been successfully used for connectomics[32,45]. However, to enable directed targeting of peroxidases to reducing environments, like the cytosol, a novel group of peroxidases was required. This motivated the engineering of an ascorbate peroxidase (APX) from the pea[46], a constitutional homodimer that is active when expressed in the cytosol. This molecule was subsequently modified to be active in its monomeric form (mAPX – monomeric APX)[46] and mutated to increase its activity (APEX – for enhanced APX)[46]. Although APX and APEX have been key for biological discoveries, including the proteomic mapping of the mitochondrial matrix[47,48] and the intermembrane space[49], its low sensitivity appeared to be a major limitation for connectomics[31] and proteomics[50]. Using a directed evolution approach to improve APEX, a mutation was found that confers improvements in kinetics, thermal stability and resistance to hydrogen peroxide concentrations. This new probe is known as APEX2[50] (Fig. 1 B1-2, E).

Contrary to HRP, neither APEX2 nor its predecessors show any indication that their activity is affected if expressed in different compartments. HRP, however, is currently still the GETEM with the highest activity when targeted properly, like to the synaptic cleft. This has motivated the development of protein-fragment complementation assays based on HRP. In these assays, two proteins of interest are fused to complementary fragments of a reporter protein. The activity of the reporter is then only reconstituted when both fragments interact. This strategy has been first

developed to visualize synaptic interactions based on two non-fluorescent fragments of green fluorescent protein (GFP). When these fragments are expressed in two distinct neurons that form synapses, they self-assemble in the synaptic cleft and fluoresce[7,51]. Using a similar approach, a split version of HRP (sHRP) has been engineered, allowing a targeted analysis of synaptic interactions in electron microscopy[52] (Fig. 1 D). A large palette of these peroxidases, including endoplasmic reticulum tagged HRP (erHRP)[31,44], vesicle tagged HRP[32], cytosolic APEX2[31] and cytosolic APX[31], have already been shown to be functional and useable for connectomics in mice and *Drosophila.*

Phosphatases

In the 1950s phosphatases naturally expressed in the epithelium of the intestinal mucosa were used to visualize the location of these enzymes in electron micrographs[53]. This was possible because phosphatases can be used in combination with β-glycerophosphate to generate a staining that is visible as a dense granular precipitate when post stained with heavy metals. In particular, the human placental alkaline phosphatase (hPALP) has played a prominent role in genetically targeted anatomical studies. It was first engineered to analyze retinal cell lineages using light microscopy[54], but soon after shown to be effective in an electron microscopic study too[55-57] (Fig. 1 F). It is important to emphasize that this was the first time a genetically encoded probe was successfully used to study neuronal networks in electron microscopy, a breakthrough celebrated as "visionary transgenic" at the time[58]. hPALP has not been routinely used in the past decade, probably because of its development preceded the innovations in serial section electron microscopy and due to the high expression level required to obtain strong staining (50 copies of the transgene were present in a single chromosomal insertion in the published studies).

Photo-oxidation

The last family of GETEM generate singlet oxygen species ($^1O_2$) upon light illumination that drive the polymerization of DAB. The first of these approaches was to use recombinant proteins that contain a tetracysteine tag, which could be labeled with a biarsenical fluorophore. This fluorophore, called ReAsH[59], was shown to be effective to polymerize DAB upon light illumination. Due to the modest $^1O_2$ quantum yield, the requirement of antidotes to prevent cell toxicity and the non-specific interactions, ReAsh has not been applied to multicellular organisms. A similar, but completely genetic targetable mechanism was later shown to be functional using the ubiquitous GFP. This method uses oxygen radicals generated during GFP bleaching to photo-oxidize DAB[60]. Unfortunately, the $^1O_2$ quantum yield of the GFP molecule is much smaller than ReAsh and it has not been shown to be functional in intact organisms. The most promising GETEM that is based on photo-oxidation is "mini singlet Oxygen Generator" (miniSOG)[61], a fluorescent flavoprotein engineered from *Arabidopsis* phototropin 2. It is a small protein of only 106 amino acids that has a substantially better $^1O_2$ quantum yield than all other GETEM. Moreover, miniSOG has been shown to be functional in *Drosophila*, where it has been successfully targeted to the cytosol, vesicles, membranes and mitochondria[30] (Fig. 1 G-H). Thus, the requirement for light is not a limiting factor for small brains. However, when larger tissues are being studied, light penetration can become restrictive.

Analysis

Visualizing a neuronal structure of a distinct cell type in an electron micrograph is challenging and in many cases impossible to define with certainty. GETEM can change this limitation by clearly defining

the structures of interest, targeting imaging efforts to defined regions of interest and aiding automatic reconstruction algorithms. A reconstruction pipeline called ARTEMIS (assisted reconstruction technique for electron microscopic interrogation of structure)[31] summarized all possible advantages GETEM can provide (Fig. 2). The first advantage is specific to the ATUM[10] technique that allows multiple and consecutive scanning of the same sample sections. This enables us to screen samples to determine regions of interest. In addition, it allows to generate overview datasets at comparative low resolution (~30 nm/pixel) relatively quickly (Fig. 2A-C). Combined with the strong contrast of cytosolic electron-dense precipitate, these overview datasets enable the reconstruction of the primary branches of the targeted cells. These reconstructions can then be used to enable targeted high-resolution imaging of the region of interest, reducing the overall imaging time and efforts. The second advantage is the segmentation and reconstruction of GETEM targeted cells by algorithms that do not require computationally intense pre-training. The primary advantage of this approach is processing time, being two orders of magnitude faster than approaches based on convolutional neural networks for membrane classification[20,26,31]. Approaches based on convolutional neural networks require adjusting thousands of parameters for each new sample, whereas algorithms developed specifically to reconstruct DAB-stained structures take advantage of unsupervised components that can be tuned with a small and fixed set of parameters. In other words, these new algorithms can be applied to all samples with only minor modifications (Fig. 2D-E).

The disadvantage of using a cytosolic staining is that the dense reaction product obscures most of the ultrastructures inside the cells, especially the presynaptic active zones. Thus, the identification of synapses made by these tagged neurons has to rely on the postsynaptic membrane specialization and the visualization of vesicles, which appear contrast-inverted due to the lack of DAB-staining inside them[31]. This is different for synapses made onto the tagged neurons. For these synapses, the presynaptic, but not the postsynaptic, machinery can be clearly visualized. For many scientific questions, these constrains do not appear to be overly limiting. For other questions, however, these constrains are a significant limitation, since in many systems the contact and synapse areas are not strongly correlated[62]. In several past studies, synaptic contacts have been inferred by only the area of contact[21,22]. These limitations can be overcome by directing the expression of the tag, either to the endoplasmic reticulum, the extracellular membrane or to the mitochondria[30,31,44]. However, the cost to be paid will be reflected in the analysis of the experiment, since overview datasets might not be as informative and the automatic reconstruction not possible. Depending on the question, a combination of complementary GETEM targeted to different neuronal populations might be the fastest approach to assess connectivity patterns in particular circuits, as shown for mitochondrial tagged APX and membrane tagged HRP[63]. Therefore, the correct choice of GETEM is bound to the experimental question and must be assessed individually.

Technical limitations of GETEM

There is no magic GETEM bullet. The strategy used will vary depending on the species, the tissue and the question being asked. Therefore, the experimental parameters must be optimized accordingly to take full advantage of the tools. The main goal of this optimization is to preserve the activity of the probes, such that a clear and unambiguously signal can be identified. This is not always trivial. Due to the fast imaging speed required to scan the ever-larger becoming volumes, it is indispensable to maximize the signal of the tissue. This is accomplished by staining protocols that take advantage of several rounds of heavy-metal staining steps to enhance the signal of the complete ultrastructural

machinery. To be able to clearly, efficiently and reproducibly identify the signals generated by GETEM, these signals must stand out from the highly-stained ultrastructure. Thus, we will next discuss the technical limitations of these probes.

Tissue fixation

To ensure that the ultrastructure of brain tissue is of the required quality for connectomics, harsh fixation protocols are needed. These fixation strategies, however, also affect the properties of GETEM. It is therefore crucial to optimize the parameters accordingly. Optimization is dependent on the GETEM being used and on the tissue being studied, requiring individual assessment in each case. The most common chemical fixation used to preserve an ultrastructure of excellent quality uses aldehydes to crosslink the tissue. Other fixation procedures, like high-pressure freezing with subsequent freeze substitution, are not appropriate for GETEM because the DAB polymerization is not possible. For aldehyde fixation, paraformaldehyde (PFA) and glutaraldehyde (GLU) are the fixative of choice. It is known that the smaller PFA can penetrate and fixate the tissue faster than GLU and is thus required to effectively preserve ultrastructure. However, the enzymatic activity of some GETEM is more prone to be affected by PFA fixation. A solution envisioned in the past is a combination of both fixatives. PFA fixation is known to be reversible and can thus be used as a first fixation step when followed by a prolonged GLU fixation without dramatically altering the GETEM activity[31].

DAB polymerization

The functionality of the GETEM can be assessed by testing the DAB polymerization efficiency. The strength of the polymerization is dependent on the concentration of the educts, the temperature and the duration of the polymerization. These three parameters can be changed to maximize the polymerization rate. It is important, however, to keep the polymerization as mild and short as possible. A prolong polymerization will oxidize the functional groups of the DAB polymer to a similar extent than the polymerization itself. It is crucial to point this out, since the chemistry that gives this polymer its characteristic brownish color and photon-density is not the same that renders it reactive to osmium. Photons are absorbed by the polycyclic structure of the polymer; osmium tetroxide reacts with the functional groups of the polymer. This affinity is higher the lower the oxidative states of the carbon atom[64]. Thus, if these functional groups are completely oxidized, the affinity of osmium to the DAB polymer would vanish[31]. In addition, and especially when peroxidases are being used, care must be taken when deciding the parameters of the polymerization reaction. First, peroxidases use hydrogen peroxide as an oxidizing component. At high hydrogen peroxide concentration, the ultrastructure is damaged. Second, the naturally expressed peroxidases in the tissue will increase the background signal and affect the quality of the ultrastructure by increasing the background.

To evaluate the efficiency of the reaction it is recommended to perform a visual inspection of the reacted tissue. This can be easily done using a ~ 20x magnification with a bright field microscope when the GETEM are localized to the cytosol or to other larger compartments, like the endoplasmic reticulum. As a rule of thumb, if the signal is clearly visible, good contrast at the EM level can be expected. As previously mentioned, the integrity of a visible precipitate does not directly imply that

the polymer could be in the adequate state to react with osmium. However, it has been shown that a mild reduction step using sodium hydrosulfite directly after the polymerization can revert the oxidative state of the polymer strongly enhancing the affinity to osmium[31].

Expression

There are two important points regarding the expression level to be kept in mind. The first one is concerning the lowest expression level required to reliably detect a signal. For some experimental designs, an unambiguous identification of all the targeted cells is necessary, whereas for others it is not. Since the strength and reliability of the precipitate is correlated with the expression level of the GETEM, the delivery, incubation and expression methods must be adapted accordingly. E.g., when using viral delivery methods, the expression times can be prolonged or the serotype changed. The second point to keep in mind concerns the highest possible expression level that does not affect the anatomy, connectivity or health of the expressing cells. So far, there is no evidence of abnormal connectivity due to the expression of genetically encoded tags[31]. However, when targeting these constructs, it is worthwhile to analyze the first order connectivity statistics of expressing cell and their surrounding neuropil. The ideal control would be to compare the motifs and connectivity statistics of two genetically defined cells, one with and the other without any tag. This control is however very difficult, since the sole purpose of the method is to simplify the identification of genetic cell types in electron microscopic data.

Suboptimal Ultrastructure

As discussed previously, the quality of the ultrastructure is affected by the dense precipitate, especially when directed to the cytosol. Although this can be overcome by targeting the GETEM to subcellular compartments, other limitations are bound to the staining protocol. This is especially the case for the visualization of gap-junctions, which are generally difficult to visualize in EM images. Gap-junctions have been shown to be essential components in vertebrate[65], as well as in invertebrate circuitries[66], making them indispensable components to model circuit dynamics. Staining protocols envisioned in the past can enhance these structures[67]. However, these protocols cannot be modified to be combined with the contrast enhancing staining protocols required for fast imaging speeds. One possibility to gather indirect evidence for gap-junctions is the preservation of the extracellular space using high-sucrose protocols[68]. Another one could be to directly tag these molecules with GETEM, but this is still work in progress.

Untapped possibilities

Combining GETEM with genetic tools that can either modify or monitor neuronal dynamics[69] is probably the most exciting future use of these tags. These combinations provide new possibilities to assess principles of connectivity and their role in computations. Such experiments have been elegantly performed in the past using purely physiological techniques[70,71] or in combination with electron microscopy[14,22,28]. These experiments have been remarkably challenging, either because of the difficulty of strenuous multi-patch electrophysiological experiments[70], sometimes combined with additional imaging approaches[71], or by the heroic effort of reconstructing the neuronal populations monitored using Ca^{2+} imaging approaches. By combining the strength of GETEM with genetically encoded reporters of neuronal activity, these types of questions could be facilitated enormously by utilizing the reconstruction of the imaged cells. Moreover, combining GETEM with immediate early

gene expression strategies[72] could prove to be useful to dissect the structural changes in plastic circuitries.

Figures and Captions

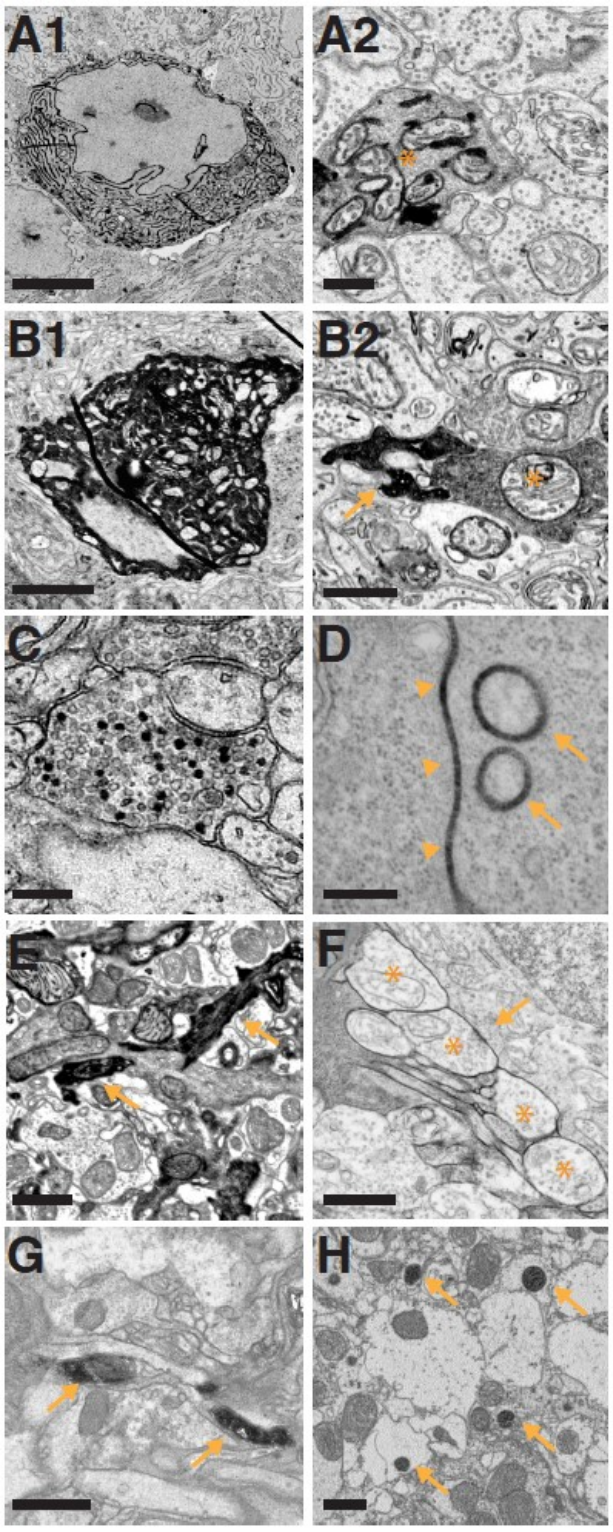


Fig 1. Genetic encoded tags for electron microscopy. A1. EM-micrograph of a mouse retinal ganglion cell soma expressing HRP in the endoplasmic reticulum and rendered electron dense. **A2**. Close-up of a ganglion cell dendrite in the retina expressing erHRP (asterisk)[31]. **B1**. EM-micrograph of

a mouse retinal ganglion cell soma expressing cytosolic APEX2 staining. **B2**. Close-up of two cytosolic APEX2 expressing processes in the inner plexiform layer (arrow and asterisk). The differential strength of the staining represents the differential amount of APEX2 expressed in each process via AAV infection[31]. **C**. EM-micrograph showing electron-dense labeled synaptic vesicles of POMC (proopiomelanocortin) positive neurons projecting from the hypothalamus. The labeling specificity was achieved by fusing HRP to the C terminus of vesicle-associated membrane protein 2 (VAMP2)[32]. **D**. sHRP staining at the contact site of two HEK293T cells: one cell is expressing the first subunit of sHRP fused to neurexin and the other cell the second sHRP subunit fused to neuroligin[48]. sHRP staining can be seen at the cell-cell junction (arrowheads), but also in internalized double membrane vesicles (arrows). **E**. APX labeled processes of direction selective neurons of *Drosophila* (arrows)[31]. **F**. Micrograph showing an electron-dense product confined to the intercellular spaces surrounding dopaminergic dendrites (asterisks) in mouse retina. One of the processes is presynaptic to the soma of an amacrine cell (arrow)[55]. **G-H**. EM-micrographs showing dendrites of projection neurons in *Drosophila* labeled with miniSOG targeted to either the cytosol (**G**) (arrows) or mitochondria (**H**) (arrows)[30]. Scale bar: A1, B1: 5 μm; A2, C: 500 nm; B2: 1 μm; D: 300 nm; F: 3 μm; E, G-H: 1 μm.

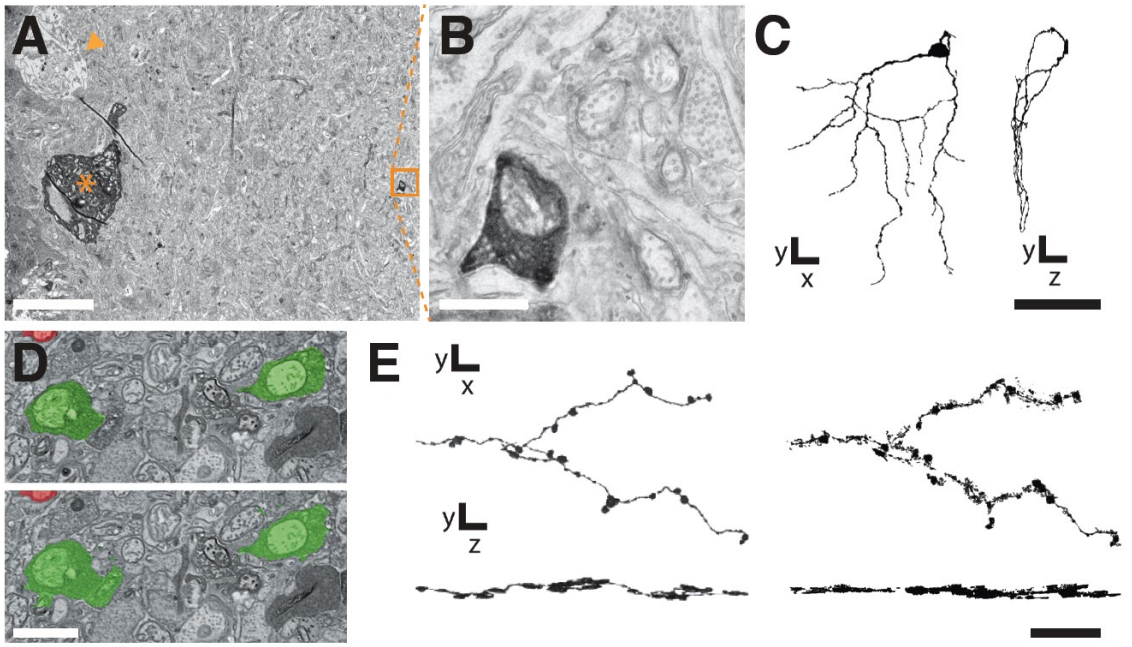


Fig 2. GETEM innovations. GETEM enable fast and unequivocal identification of genetic identified cell types in EM-micrographs. **A**. Overview resolution EM-micrograph of a retinal section (30 nm / pixel) depicting an unlabeled (arrowhead) and an APEX positive somata (asterisk), as well as a small dendritic process (box). **B**. Enlarge view of an APEX positive process. **C**. The high contrast of these processes assists the reconstruction of the main cellular processes, as represented by the reconstruction of a retinal ganglion cell (J-RGC) from a similar data set shown in **A-B**[31]. These reconstructions can be used to concentrate high-resolution imaging efforts to particular regions of interest. **D**. High-resolution EM-micrograph depicting APEX positive processes of an interneuron in the retina, the so-called starburst amacrine cell. Unsupervised automatic segmentation algorithms can identify labeled processes with acceptable error rates compared to manual segmentation. Ground-truth, manual segmentation (top); automatic segmentation (bottom). **E**. Manual (left) and

automatic (right) reconstruction of a starburst amacrine cell process. *En face* (top) and side view (bottom)[31]. Scale bars: **A**: 10 µm; B: 1 µm, **C**: 50 µm; **D**: 10 µm; **E:** 10 µm.

Conclusion

Our understanding of the brain's function is based to a large extent on the anatomical characterization of the nervous system, most prominently exemplified by the unmatched contribution of the detailed schematic drawings by Cajal. Using the Golgi staining method, a sparse labeling technique, Cajal not only determined the building blocks of our brain but also defined the way we think about the function of circuits[73,74]. Connectomics, as a field, intends to use Cajal's conceptual methodology and map the detailed connectivity diagrams at ultra-resolution. The goal is that these connectivity diagrams reveal structural constraints that can verify or refute computational models, as already shown in the past[14,20,22,28,29,75]. Although structural wiring details *per se* are insufficient to derive circuit dynamics[76], they can contribute immensely to the functional understanding of the brain in health and disease. GETEM provide a useful toolbox that, as seen with the fluorescent toolbox in the last two decades[77], could also revolutionize the study of biological structures. Finally, as with Cajal's first steps, the field of connectomics endeavors into an unknown territory[78]. Exploring the least understood and most complex system we know, the brain, will doubtless provide unexpected, eye-opening surprises.

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