A correlative array tomography protocol to investigate projection neuron circuits

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A CORRELATIVE ARRAY TOMOGRAPHY PROTOCOL TO INVESTIGATE PROJECTION NEURON CIRCUITS

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Abstract

Information about synaptic connectivity and three-dimensional structure of neurons is fundamental to understand neural microcircuits. Unfortunately acquisition of this information remains challenging, because of the relatively large volumes which need to be imaged at high resolution. Among different microscopy approaches based on light and electrons, a promising technique is array tomography, in which arrays of ultrathin brain sections are stained with fluorescent antibodies against neurotransmitters and synaptic proteins and are imaged in light and electron microscopes (Micheva and Smith, 2007).

Based on array tomography, we developed a correlative microscopy approach to study projection neurons between different brain regions in the zebra finch. We inject fluorescent neural tracers of different colors into areas afferent and efferent to the region of interest. After preparation of the tissue for electron microscopy, we prepare arrays of serial ultrathin sections which we image in the light microscope, either directly after sectioning, or after performing immunolabeling with anti-dye antibodies. The same sections are then imaged in the electron microscope. Fluorescence signal allows to classify synapses and neural processes identified in electron microscopy imagery. Thanks to a tissue preparation which includes fixation and staining with heavy metals, high membrane contrast and ultrastructure quality is achieved in the electron microscope. Good ultrastructure and reliable fluorescence information allow us to characterize synapses made by thalamic neurons in the forebrain premotor area HVC, and in the same tissue a different fluorescence channel allows us to investigate neurons projecting to the motor pathway. The application of our preparation method will be useful to investigate the morphology of neurons and synapses belonging to different populations and will provide statistical information about their connectivity, which is crucial to understand how signals in the brain are routed among different areas and transformed into meaningful motor output.

In addition to this main part, we present additional experiments we conducted to explore preparation methods, which avoid the use of heavy metals (the cause of strong fluorescence reduction during embedding), and screening experiments
to identify adeno-associated viral vectors suitable for neural labeling in the zebra finch.
La conoscenza della connettività a livello sinaptico e della struttura tridimensionale dei neuroni è fondamentale ai fini della comprensione di circuiti neurali. L’acquisizione di questo tipo di informazione è tuttavia difficoltosa, a causa della risoluzione necessaria e dei volumi relativamente grandi che devono essere fotografati. Tra le differenti strategie basate su microscopia ottica ed elettronica, una tecnica promettente è rappresentata dall’*array tomography*, in cui serie di sezioni ultrasottili di cervello vengono colorate con anticorpi fluorescenti contro neurotransmettitori ed altre proteine sinaptiche e fotografate con microscopi ottici ed elettronici (Micheva and Smith, 2007).

Basandoci sull’*array tomography*, abbiamo sviluppato un approccio di microscopia correlativa per studiare neuroni di proiezione tra diverse regioni del cervello del diamante mandarino. Iniettiamo nelle regioni afferenti ed efferenti alla zona studiata elementi traccianti fluorescenti di diversi colori. Dopo aver preparato il tessuto per microscopia elettronica, prepariamo serie ininterrotte di sezioni ultrasottili che fotografiamo nel microscopio ottico, direttamente dopo averle tagliate o dopo avere eseguito una colorazione con anticorpi fluorescenti nel caso di perdita della fluorescenza delle sostanze traccianti durante la preparazione. Fotografiamo in seguito le medesime sezioni con il microscopio elettronico. Il segnale fluorescente permette di classificare sinapsi ed altre strutture neurali identificate nelle immagini acquisite con il microscopio elettronico. Grazie ad una preparazione del tessuto che include fissazione e colorazione con metalli pesanti, un’alto contrasto delle membrane e qualità ultrastrutturale sono visibili delle micrografia elettroniche. Mostriamo come la buona ultrastruttura e l’affidabile informazione fluorescente permettono di caratterizzare sinapsi di neuroni provenienti dal talamo nell’area prefrontale HVC e come nello stesso tessuto un diverso canale fluorescente permette di investigare neuroni che proiettano in zona motoria. Il nostro metodo di preparazione sarà utile per investigare la morfologia di neuroni e sinapsi appartenenti a diverse popolazioni e darà informazioni statistiche sulla loro connettività, cruciale per comprendere come segnali nel cervello vengono trasmessi tra varie aree e trasformati in un output.
motorio sensato. In aggiunta a questa parte principale, presentiamo esperimenti che abbiamo condotto per esplorare metodi di preparazioni che evitano metalli pesanti (la causa della grande riduzione di fluorescenza durante la preparazione del tessuto) ed esperimenti di screening che per identificare vettori basati su virus adeno-associati per marcare neuroni nel diamante mandarino.
Chapter 1

Introduction

1.1 The songbird and the need of connectivity information

Electrophysiological and optical recordings of neural activity have greatly contributed to our understanding of how neural networks function. The songbird species zebra finch (*Taeniopygia guttata*, Fig. 1.1) is an excellent example of an animal model in which electrophysiological studies have shed light on the activity patterns in areas involved in song learning and production (Mooney, 2009). Male zebra finches have the interesting property of being able to imitate the highly stereotyped song that they hear from their tutors, in contrast to most animal species in which vocalizations are the result not of a learning process, but of an innate repertoire of sounds. Learning occurs in two phases: during a sensory phase, the juvenile animal listens to the tutor and memorizes its song, while in a later sensorimotor phase the juvenile vocalizes and, according to a model, uses auditory feedback to match its own song with the memorized tutor song (Konishi, 1965).

The song control system

A specialized set of brain areas is involved in song learning and production (some of these are shown in Fig. 1.2). One of these is HVC (used as a proper name), a premotor area located in the dorso-posterior part of the forebrain which also receives auditory information. HVC gets, among other projections, input from the nucleus interface of the nidopallium (NIF) and from the thalamic nucleus uveaformis (Uva), and sends projections to the basal ganglia nucleus Area X, involved in generating song variability (Scharff and Nottebohm, 1991; Reiner et al., 2004; Olveczky, Andalman, and Fee, 2005), and to the robust
nucleus of the arcopallium (RA), which in turn relays information to the motoneurons of the vocal organ and to respiratory areas. Individual HVC neurons projecting onto RA neurons (HVC\textsubscript{RA} neurons) have been shown to produce bursts of spikes sparsely, at single, precise time during the song motif, with individual neurons bursting sequentially with respect to one another (Hahnloser, Kozhevnikov, and Fee, 2002). These bursts drive RA, whose neural activity is more dense (more than 10 bursts per neuron) and in which different RA ensembles produce similar song elements, with patterns of neural activity changing on a short timescale (5-10 ms), in contrast to the modulation of the song which occurs on a wide range of time scales (10-100 ms) (Leonardo and Fee, 2005).

The song is finally produced by the air flow through the syrinx, the vocal organ which is located at the conjunction of the trachea with the bronchi. The syringeal muscles are controlled by motoneurons located in the brainstem in the tracheosyringeal part of the hypoglossal nucleus (XIIts), which receives direct projections from RA (Roberts et al., 2007). Thus, within few synapses, the sparse representation of the song observed in HVC, in which individual RA-projecting neurons produce a single burst of spikes during a song motif, is transformed into continuous activity of the syringeal muscles, passing through a still discrete but more dense (compared to HVC) activity in RA.

Electrophysiological observation of the activity in these areas has led to a simple model of vocal sequence generation (Fee, Kozhevnikov, and Hahnloser, 2004; Fee and Scharff, 2010). According to this model, HVC neurons generate a continuous sequence of activity over time, with a small group of HVC projecting neurons active at a time point, and not at any other time. These neurons in turn drive a burst of activity in a subgroup of RA neurons (around 10%), with the exact ensemble determined by the pattern of synaptic connectivity between HVC projection neurons and RA neurons. Finally, the input from RA neuron

![Figure 1.1: A male zebra finch.](image)
ensembles converges on a short time scale of about 10 to 20 ms to single motoneurons of Xlls, which control the configuration of the syrinx. Activity could propagate through HVC like a wave (Long, Jin, and Fee, 2010) and the nucleus could thus act as a clock controlling song timing. This last hypothesis has been tested by cooling HVC and observing that it results in slowing the song speed across all timescales, but only slightly altering the acoustic structure, whereas cooling the RA has no observable effect on song timing, thus reinforcing the idea that song timing is determined by HVC (Long and Fee, 2008).

From HVC, another projection goes to the anterior forebrain pathway, which includes Area X, the thalamic nucleus DLM (medial portion of the dorsolateral thalamus), and LMAN (lateral magnocellular nucleus of the anterior nidopallium), which in turn projects to RA. This pathway has been shown to be crucial for song learning in juveniles, as lesions in Area X and LMAN prevent learning (LMAN lesion results in monotonous repetitions of a single note complex, Area X lesions in rambling series of unusually long and variable notes), while they have little effect on adult singing ability (Scharff and Nottebohm, 1991; Fee and Scharff, 2010).

Anatomical characterization of HVC neurons

Neurons projecting to Area X (HVC\textsubscript{X} neurons) and HVC\textsubscript{RA} neurons have been characterized at the LM level in the past. Similar to HVC\textsubscript{X} neurons, HVC\textsubscript{RA} neurons have extensive axon collaterals within HVC (Mooney, 2000), but have been described to be smaller both regarding somata size (surface area of around 105 \(\mu m^2\) for HVC\textsubscript{RA}, 145 \(\mu m^2\) for HVC\textsubscript{X}) and maximal extension of the dendrites (around 200 \(\mu m\) for HVC\textsubscript{RA} neurons, 250 \(\mu m\) for HVC\textsubscript{X} (Dutar, Vu, and Perkel, 1998)). In addition to the smaller somata size and more compact dendritic arbour, HVC\textsubscript{RA} somata tend to have more rounded profiles than HVC\textsubscript{X}
and interneurons, and to have thinner and more sparsely spined dendrites compared to the densely spined \( \text{HVC}_X \) dendrites (Mooney, 2000). Some authors have also distinguished two classes within \( \text{HVC}_{RA} \) neurons, one with small somata and lightly spined dendrites, the others with medium-sized somata and thick, densely spined dendrites (Fortune and Margoliash, 1995).

In the adult canary HVC, neural somata tend to be distributed in clusters, often composed of both \( \text{HVC}_{RA} \) and \( \text{HVC}_X \) projection neurons, with a single \( \text{HVC}_X \) neuron surrounded by many \( \text{HVC}_{RA} \) neurons (Kirn et al., 1999). In the same species, electron microscopic analysis of HVC confirmed the presence of tight neuron clusters, with neurons directly contacting adjacent somata, although the identity of the neurons composing these clusters could not be assessed (the authors only labeled newly generated neurons with \( ^3 \text{H}-\text{thymidine} \)) (Burd and Nottebohm, 1985). It has been speculated that the tight contact of neighboring cells might be due to the presence of electric synapses. Gahr and Garcia-Segura analyzed with freeze-fracture EM HVC of testosterone-treated female canaries, finding indeed soma-somatic gap junctions and a dependence of the number of gap junctions on the testosterone level (Gahr and Garcia-Segura, 1996). It is interesting to note that dye coupling (labeling of a second cell body after a single somatic injection) has been observed in zebra finch HVC cells, possibly due to coupling of the cells via gap junctions (Dutar, Vu, and Perkel, 1998).

**Thalamic input to HVC**

Among the inputs received by HVC, the thalamic nucleus Uva has been shown to provide a direct linkage between the auditory brainstem and HVC, gating auditory responses (Coleman et al., 2007; Hahnloser et al., 2008). Uva neurons have been shown to be responsive to visual and to somatosensory stimuli (Wild, 1994). Moreover, robust auditory activity has been found in Uva, possibly due to its innervation from the ventral nucleus of lateral lemniscus (an auditory brainstem component), although it appears that Uva does not elicit auditory responses in its targets, as silencing of Uva has little effect on auditory activity in HVC neurons (Coleman et al., 2007). In HVC, as well as in other vocal control nuclei, auditory response to the bird’s own song has been observed, and the responsiveness and selectivity of the response has been found to be modulated by the behavioral state of the animal (Schmidt and Konishi, 1998). High-frequency stimulation in Uva can suppress auditory activity in both HVC and NIf (the two direct targets of Uva), suggesting that Uva can gate auditory responses in HVC (e.g., by withdrawing excitatory drive from NIf, which directly projects to HVC, or through a mechanism involving inhibition local to HVC) (Coleman et al., 2007). This role in gating is supported by the fact that Uva receives
cholinergic input from the neurons in the medial habenula and afferents from the nucleus reticularis superior, pars dorsalis, thalamic areas which have a neuromodulatory role in other vertebrates (Akutagawa and Konishi, 2005). Experiments in the sleeping zebra finch have shown that single spikes in Uva_{HVC} neurons mediate inhibition in HVC, whereas bursts in Uva_{NIf} neurons mediate excitation in NIf (Hahnloser et al., 2008). It has been suggested that Uva may play a role in bilateral coordination. In the songbird forebrain, no bilateral connections have been found, despite singing being controlled by both hemispheres (Wang et al., 2008). Uva however receives bilateral projections from the nucleus paraambigualis, involved in inspiration, and from the vocal dorsomedial nucleus of the intercollicular complex (DM) (Ashmore, Wild, and Schmidt, 2005; Schmidt, Ashmore, and Vu, 2004). It has been suggested that bilateral coordinated Uva_{HVC} activity may mediate interhemispheric synchronization, for example by suppressing HVC activity before singing (Hahnloser et al., 2008; Wang et al., 2008). A second input to HVC comes from NIf, a nucleus located in the auditory Field L region, which has been shown to be an auditory afferent to HVC and also provide motor input (Coleman et al., 2007; Naie and Hahnloser, 2011).

Open questions in songbird connectivity

Whereas on one side electrophysiological recordings, together with lesion, pharmacological, and tract tracing studies, allow to model the connectivity of these networks, which underlies their behavior, these models could be considerably constrained by the knowledge of the actual synaptic connectivity, the wiring diagram, of the networks. If we consider for example thalamic input coming from Uva to HVC, we do not know the synaptic targets of Uva_{HVC} neurons in HVC, whether they target interneurons or projection neurons, and, if the second case is true, which populations and in which proportion. If we consider RA-projecting HVC neurons, we do not know if they actually form a chain connectivity as expected from sequential activity in neurons observed in electrophysiological recordings, and we do not know how these neurons interact with neurons projecting to Area X and the anterior forebrain pathway. Similarly, more downstream, we do not know if motoneurons in nXIIIts controlling different syringeal muscles do not make monosynaptic contacts between each others, similar to motor pools in the spinal cord of other animal models (Stepien, Tripodi, and Arber, 2010).
An example of connectivity investigation

A reason for the lack of detailed anatomical knowledge is that this information can only be gained from imaging of the brain at a resolution high enough to identify synaptic connections between neurons, and this requires in most cases electron microscopy (EM), which is a highly time consuming method (Briggman and Denk, 2006). A relatively complete map of the connectivity has been so far only elucidated for the nervous system of one animal, the nematode *Caenorhabditis elegans* (White et al., 1986; Varshney et al., 2011). Motivated by the idea that the functional properties of a nervous system are largely determined by the morphology of its component neurons and the pattern of synaptic connections between them, White et al. deduced the structure and connectivity of the hermaphrodite nervous system based on a complete reconstruction from electron micrographs of serial sections (only neurons in the pharynx were not reconstructed). They identified 302 neurons, which they classified depending on the morphology and connectivity, and about 5000 chemical synapses. This pioneering work laid the groundwork for a multitude of successive studies. Because of the consistency of the nervous system between different *C. elegans* individuals, it has been possible to use the reconstruction and resulting wiring diagrams to plan experiments and interpret results (see as an example the study by (Mori and Ohshima, 1995) about neural regulation of chemotaxis, but many other experimental and theoretical works could be cited).

Unfortunately, due to the amount of work needed to prepare, image and reconstruct neural tissue, studies in which electron microscopy was applied to investigate neural connectivity have been mostly limited to small parts of neurons or of the neuropil. In recent years, however, development of informatics tools and automation methods in electron microscopy imaging have allowed speeding up the whole process. In the next sections, we will give an overview of some of the current approaches to reconstruction of neural tissue, which are based on electron microscopy, as well as on light microscopy or combinations of both.

1.2 Electron microscopy based reconstruction methods

EM based methods take advantage of the resolution that can be achieved with electron microscopy, which is sufficient for the structures that need to be imaged to investigate neural circuit connectivity. These can have a size of hundred nanometers or smaller, as it has been for example reported for axons in the rat hippocampus (Shepherd and Harris, 1998) or for axons projecting from the claustrum to the cat primary visual cortex, which can have a diameter of 60 nm...
Moreover, synapses can be directly identified without additional need of specific staining, because some of their components, such as synaptic vesicles, specialized pre- and post-synaptic membranes, synaptic cleft and postsynaptic density, are directly visible in chemically fixed, osmium tetroxide (OsO$_4$)-treated and resin embedded tissue, also providing information about their polarity. In fact, it was with transmission electron microscopy (TEM) that it could be unequivocally shown in the 50’s of the last century that neurons communicate via synaptic junctions, and it was possible to start classifying them based on their morphology, for example thickening of the pre- and postsynaptic membrane and synaptic vesicle shape (Gray, 1959).

Crucial for neural circuit reconstruction is preservation and visibility of membranes, to be able to trace neurons and distinguish synaptic components. This contrast is normally achieved by fixation and staining of the tissue with heavy metals, mostly osmium tetroxide (OsO$_4$), uranyl acetate and lead citrate, which result in accumulation of the heavy metals, which are electron dense and thus give contrast in the EM, on the structures of interest. The stained tissue is then dehydrated and embedded in a resin (see chapter 2.2.2 for a discussion about preparation methods), which can be cut with a diamond knife and results in ultrathin sections which can be imaged in the EM.

1.2.1 Serial section transmission electron microscopy

The approach used to reconstruct the *C. elegans* nervous system consisted in sectioning resin-embedded brain tissue in ultrathin sections (50-80 nm) using a diamond knife, collecting these on films supported by slot grids, imaging them individually in a TEM, post-processing the images by registering serial sections, segmenting them and reconstructing the neurons inside them (Harris et al., 2006). Systematic approaches to the handling and post-processing of large numbers of images, including computers to aid in the collection of the three-dimensional structure of neurons, were already used in the 70’s (Macagno, Levinthal, and Sobel, 1979). The typical workflow involved production of film strips of aligned serial sections, in which the frames of the film were pictures of the sections in the correct sequence. Viewing the film at fast speed allowed the researcher to get a sense of the three-dimensionality of the structures. Additionally, moving a tablet with a pointer connected to a computer on the images allowed recording the x-y position of a structure of interest, with the z position calculated based on the frame being analyzed. Digitized coordinates could then be used to construct three-dimensional models of neurons or to investigate distributions of synapses. The approach of serial section transmission electron microscopy (da Costa and Martin, 2009).
microscopy (ssTEM) suffers of the drawbacks of requiring large amount of manual work, mainly to collect and image large numbers of ultrathin sections, a procedure which is prone to error, and the related difficulty to reliably produce long sequences of consecutive sections without section loss. Additionally, micrographs acquired with ssTEM need to be aligned, because sections lie with different orientations on consecutive grids and can have different degree and direction of cutting distortion, and the thickness of the sections that can be cut is limited to several tens of micrometers. Still, ssTEM has the advantages of very high lateral resolution, of the ability to tilt sections in the microscope enabling tomography techniques, and of much higher imaging speed compared to scanning electron microscopy (SEM), which is the microscopy technique used for other approaches (see 1.2.2 and 1.2.3). For these reasons, in many cases ssTEM is still currently used to investigate neural circuits.

**Dense reconstruction with ssTEM**

An excellent example of current ssTEM-based reconstruction is the analysis of the architecture of the *Drosophila melanogaster* brain (Cardona et al., 2010). The authors prepared uninterrupted series of TEM sections of *Drosophila* brain at an early larval developmental stage, whose neuropil (located in the center of an outer cortex of cell bodies) has a diameter of 30 µm, and of part of the ventral cord nerve (in total 750 sections). Thanks to a software package, Legion, which can control every component of the EM and the camera, the authors semi-automatically acquired images of the complete sections at a resolution of approximately 3-4 nm (a resolution that allows to clearly resolve ultrastructural details such as synaptic vesicles). To reconstruct neural microcircuitry, the authors then developed and used the open source software package TrakEM2, which includes all the tools needed for data acquisition, registration, navigation and segmentation. The reconstruction allowed the authors to classify neurites based on their morphology and synaptic properties (for example presence of presynaptic or postsynaptic sites on neurites with specific shapes), and to describe some network motifs of the *Drosophila* neuropil, which could then be compared (for example on a statistical level) to the vertebrate nervous system. Another example of a comprehensive description of the circuitry and synaptic connectivity of a neural circuit using ssTEM is the analysis of the rabbit retina (Anderson et al., 2011). The work is based on a framework and workflow developed by the same research group for automated TEM imaging of large-scale neural assembly (Anderson et al., 2009). The framework combines small molecule profiling with automated TEM acquisition, image tile mosaick-
ing, slice-to-slice image registration and gigabyte-scale image browsing to annotate images, similar to TrakEM2. The small molecule profiling consists in immunoprobing some of the ultrathin sections (typically the first sections of the volume) for different small molecules (e.g. GABA, glutamate, glycine, taurine, glutamine), imaging them optically, and registering the multichannel images with electron micrographs of the following sections (in other words, sections are either imaged in the LM or EM). This procedure results in molecular signatures which can be used to classify cells and create maps thereof. The workflow was used in the case of the rabbit retina to produce 16.5 terabyte of images at a resolution of around 2 nm, representing a column of tissue of 250 µm diameter spanning the inner nuclear, inner plexiform and ganglion cell layers. Whereas high-resolution electron micrographs allowed the researchers to trace neurons in the tissue and identify synapses, the molecule profiling allowed them to distinguish cells expressing different neurotransmitters and discriminate different kind of bipolar, amacrine and ganglion cells based on their molecular signatures. The analysis allowed investigating connectivity patterns of different cell types and discover novel synaptic associations. Moreover this approach based on imaging of complete volumes, similar to the *C. elegans* case by White et al. and the *D. melanogaster* case by Cardona et al., offers the opportunity for additional data mining and deeper analysis of specific networks.

A recent large-scale ssTEM project has been conducted to investigate connectivity of visual cortical neurons (Bock et al., 2011). The authors took advantage of a modified TEM with an extended vacuum chamber which allows to acquire 2 x 2 images simultaneously (using 4 high speed CCD cameras) covering a large section area. They imaged more than 1200 serial sections of a tissue (with an area of 350 x 450 µm²) which was previously imaged *in vivo* to determine the preferences for stimulus orientation of cells in layer 2/3 of the mouse primary visual cortex. Using blood vessels of successively finer caliber which were fluorescent and also visible in the EM, they identified the cell bodies of neurons of known orientation and manually traced their dendritic and axonal arbors. In this way, they were able to correlate functional information with anatomical connectivity, finding that inhibitory neurons receive synaptic input from nearby excitatory neurons with a broad range of preferred orientations.

**Neuron labeling and sparse reconstruction with ssTEM**

The approach of imaging entire volumes at high resolution is becoming popular, also thanks to other automated methods based on SEM which allow dense reconstruction of the neuropil (see 1.2.2 and 1.2.3). Acquisition and handling
of large amounts of data (such as the 16.5 terabyte of retina dataset previously mentioned) has become possible thanks to digital photography and computer-based processing of the images. A different but very prolific approach based on ssTEM consists in the reconstruction of only specific neurons in the EM, often previously labeled with electron-dense markers.

First labelings of neurons with markers visible in the EM were based on the Golgi method of metallic impregnation of neurons, a staining procedure that had allowed Ramón y Cajal to realistically reconstruct a variety of neural circuit architectures. In the 60’s of the last century, W. Blackstad demonstrated that the Golgi impregnation resulted in an electron-dense, massive and intracellular precipitate, and revealed its potential usefulness as an electron microscope marker (and, more in general, revealed the power of EM markers) by combining it with axonal degeneration (Blackstad, 1975; Fairen, 2005). He transected the perforant pathway connecting the entorhinal area with the hippocampus, which resulted in degenerated boutons distinguishable in the EM by their appearance, and also stained the tissue with the Golgi method. With these two different markers visible in the EM, Blackstad proved that entorhino-dentate fibers end on the dendrites of granule cells of the hippocampus, making asymmetric synapses, and introduced a method for cell-to-cell mapping of neural connections.

In the following years, more elaborate ways to label neurons and trace pathways with EM-visible markers were developed. An example is the axonally transported plant lectin PHA-L (*Phaseolus vulgaris* Leucoagglutinin). Lectins are plant proteins that bind to certain glycoproteins of neurons, a property that facilitates their uptake and transport by neurons, and PHA-L was found to be a sensitive, anterogradely transported marker with limited uptake by fibers of passage which could also be made visible in the EM (Gerfen and Sawchenko, 1984; Deller, Naumann, and Frotscher, 2000). Unfortunately, the PHA-L method suffers form the drawback of unreliability, and for this reason in successive years other methods to trace neurons were explored (Reiner et al., 2000). A great improvement was achieved with the use of the horseradish peroxidase (HRP), which allowed tracing neural connections retrogradely, anterogradely and, by using a modified HRP version (wheat germ agglutinin-coupled HRP), transynaptically. HRP could be made visible by a simple, easy to perform and reliable histochemical reaction (Kristensson and Olsson, 1971; Vercelli, 2000). Dextran-lysine conjugates were also found to be well suited for pathway tracing, with reliable uptake and delivery by neurons and with the ability to fix them *in situ* by virtue of lysine residue located on the dextran chain (Glover, Petursdot-tir, and Jansen, 1986; Nance and Burns, 1990). Although dextran was first introduced coupled to fluorophores, a biotinylated version, BDA (biotinylated
dextran amine), was later introduced and found to provide an extensive and detailed neural labeling, both as an anterograde and retrograde tracer. BDA could be developed with an avidin-biotinylated HRP procedure to produce an electron-dense, permanent reaction product, making connectivity study with dextrans possible at the EM, with low molecular weight preferentially used for retrograde labeling and high molecular weight for anterograde labeling (Veenman, Reiner, and Honig, 1992). In addition to BDA, HRP and PHA-L, other tracers are now used, including biocytin, neurobiotin and CTB (cholera toxin B subunit). Biocytin, in particular, can be used as an anterograde tracer (although some retrograde labeling can also occur), with fast uptake and diffusion within the cell. Biocytin is also well suited for intracellular recording, because it does not cause physiological alterations, and results in good staining of neurons, including dendritic spines (Vercelli, 2000).

BDA has since its development been used to investigate connectivity in combination with ssTEM. An excellent example is the study of thalamocortical projection in the cat visual cortex (da Costa and Martin, 2009). The authors analyzed quantitatively the thalamic input to pyramidal cells of layer 6, which are the source of corticothalamic projection and provide excitatory synapses to synapses in layer 4 (more than the thalamic input does). They injected BDA in the dorsal lateral geniculate nucleus to trace anterogradely the thalamocortical pathway and also retrogradely a small number of layer 6 corticothalamic pyramidal neurons. Using light microscopy, they reconstructed the dendritic arbors of few layer 6 cells in regions of area 17 in which thalamic afferents were also labeled. In this way, they were able to map the positions on the dendritic tree of the corticothalamic cells where thalamic input formed contacts. With ssTEM they examined these contacts and tested if they were synapses, finding that only half of the contacts were synapses and of these only some were actually synapses between the two labeled neurons, revealing the impossibility of predicting from an LM observation whether a contact is forming a synapse (because the contact is not a synapse at all, or it is a synapse but with other contacting neurons and not the one labeled). With this work-intensive anatomical reconstruction, the authors were able to unambiguously characterize strength and spatial distribution of thalamic input on corticothalamic cells, helping to clarify the role of these projections on computation in the cortex.

In another study, the same authors took advantage of the same strategy of combining LM and ssTEM to investigate thalamic connections to spiny stellate cells in the cat’s visual cortex, projections which are known to determine the response of simple cells but which make only few excitatory synapses in layer 4 (da Costa and Martin, 2011). Injection of BDA in the dorsal lateral geniculate nucleus was combined to intracellular labeling of neurons in layer 4, followed by
light microscopic identification of contacts between thalamic axons and spiny stellate dendrites. With ssTEM, the authors analyzed contacts and identified synapses at the ultrastructure level, characterizing their location, number and size, finding that thalamic axons form only a small number of synapses with spiny stellate neurons and that these are not located particularly proximal to the soma nor are they clustered on the dendrites. With this characterization, the authors were able to point to specific mechanisms to explain the efficacy of thalamic input, for example synchronous activation of the sparse thalamic synapses, and to support the idea that thalamic input does not by itself determine the cortical response of spiny stellate neurons.

A different example of neural labeling combined with ssTEM is the investigation of the dynamics of spine growth and synapse formation (Knott et al., 2006). The authors addressed the question whether in cortex new spines grow from previously existing shaft synapses or whether new spines grow to contact an axon with subsequent formation of new synapses. To answer this question, they used transgenic mice, subjected to sensory stimulation by whisker trimming, expressing enhanced green fluorescence protein (EGFP) in a sparse subset of pyramidal neurons and imaged these cells in vivo in the barrel cortex over 1 month, tracking gains and losses of spines. After perfusing the animals, they produced an electron-dense substrate in the labeled neurons using pre-embedding immunohistochemistry against EGFP and an avidin-biotinylated HRP procedure with diaminobenzidine (DAB). This EM labeling allowed reconstructing the imaged dendritic spines and surrounding neuropil using ssTEM and analyze their ultrastructure to test for the presence of synapses. The resulting data showed that new spines always had synapses if they persisted for a few days or more, whereas most of the spines reconstructed immediately after they were first seen did not have synapses, revealing that in the adult cortex spine growth precedes synapse formation.

Targeted use of ssTEM to address questions related to synaptic connectivity, as in the previous examples, is an extremely powerful tool. It however requires large amounts of manual work, in particular for questions which require dense reconstruction of big volumes, and can be limiting if a very high z-resolution is needed. Three recently developed techniques overcome these disadvantages: Automated Tape-collection Lathe Ultra Microtome (ATLUM), Serial Block-Face Scanning Electron Microscope (SBEM), and Focused Ion-Beam Scanning Electron Microscope (FIBSEM).
1.2.2 Serial section scanning electron microscopy

Automated Tape-collection Lathe Ultra Microtome (ATLUM)

The ATLUM, which is so far only a proof of principle and has not been yet used to produce scientific results, is a modified ultramicrotome developed by Hayworth and coworkers which collects ultrathin sections continuously onto a carbon-coated polymer tape (Hayworth et al., 2006). Like a conveyor belt, the tape is inserted into the waterboat of the diamond knife, and collects the ribbon of sections with same speed as they are being cut from the block. The tape can be then cut in short pieces and attached to a silicon wafer, for imaging or storage. Automation of the cutting and collection procedure has the advantage of reducing damage or loss of the sections as it can occur when a block is manually cut and the sections are collected individually or in short ribbons. Tapes containing over 1000 consecutive sections have been reported to have been collected without human intervention and without breaks (Hayworth et al., 2006), and also the section thickness which can be achieved has been reported to be lower than in ssTEM, about 25 nm (Helmstaedter, Briggman, and Denk, 2008). The ultrathin sections collected on the tape can then be imaged with a scanning electron microscope.

Another great advantage of this approach is that a library of neural tissue can be produced, stored, and imaged repeatedly. In other words, unlike other automated techniques which destroy the tissue after imaging, but similar to ssTEM, the same sections can be used many times, for example to image again a region of interest at higher resolution, or to image a different area to address a different question. Moreover, since scanning electron microscopes are still slow to the purpose of imaging large volumes at synaptic resolution, the tape containing the sections can be cut in different pieces and these can be imaged in parallel by many microscopes, thus speeding up the acquisition process. In addition sections cut with ATLUM have the benefit of being in principle easily stainable. Whereas with ssTEM staining requires manual handling of a high number of fragile slot grids (with the risk of damaging them), a tape with many sections can be placed on a rigid substrate facilitating post-embedding staining, which can either be done to increase membrane contrast or to detect specific antigens in the tissue.
1.2.3 Block-face imaging

The idea of block face imaging is to overcome some of the limitations of ssTEM by imaging sections before cutting them, i.e. by repeatedly imaging the surface of the block and removing away thin layers of tissue between acquisition of the images. Two block-face scanning methods are currently developed, one aiming at imaging large tissue volumes, the other at achieving very high z-resolution.

Serial block-face scanning electron microscopy (SBEM)

SBEM consists in an ultramicrotome mounted inside the chamber of an SEM to remove slices of tissue from the block, which is imaged after each slicing step, similar to an approach used with LM (Denk and Horstmann, 2004). Because the diamond is moved for cutting, the position of the block remains stable and subsequent images are already aligned, thus facilitating post-processing of the images. Moreover, using back-scattered electron detection and low beam energies, the penetration of the electron in the tissue can be limited to few tens of nanometers (Helmstaedter, Briggman, and Denk, 2008). SBEM has the advantage that the cutting and imaging operation does not require human intervention (or only minimal intervention to adjust imaging parameters), thus allowing to automatically collect three-dimensional datasets.

The potential of SBEM has been shown in a recent publication in which the direction-selectivity circuit of the retina has been investigated by in vivo imaging combined with volume reconstruction in the EM (Briggman, Helmstaedter, and Denk, 2011). Direction-selective retinal ganglion cells, which receive projection from starburst amacrine cells, are known to respond stronger to motion oriented along a preferred direction, but it was unknown whether the asymmetry in response was implemented at the structural level in the wiring of circuitry or resulted from unequal synaptic strength in an otherwise symmetric circuit. To address this question, the authors first characterized ganglion cells functionally, with two-photon calcium imaging, and structurally, by acquiring a 350 x 300 x 60 µm³ SBEM volume with a lateral resolution of 16.5 nm and a section thickness of 23 nm. After reconstructing several ganglion cells in the EM data by skeletonizing them, they identified connections between the cells and starburst amacrine cells and also reconstructed these (these contacts, due to the tissue preparation used to have high extracellular space staining, were not directly identifiable as synapses, but could be deduced to be so based on their shape and comparing it with conventionally prepared tissue). In this way, they were able to examine the specificity of synapses between the two neuron classes and found that starburst amacrine cells selectively synapse with a direction-selective retinal ganglion cell if they are oriented 180° from the preferred direction.
Focused ion beam scanning electron microscopy (FIBSEM)

An alternative approach to the mechanical microtomy of SBEM is using a focused beam of gallium ions directed parallel to the surface of an embedded block for milling thin layers of tissue, while a scanning electron beam in the same microscope images the milled region (Knott et al., 2008; Heymann et al., 2006). When the technique was introduced in the context of brain imaging, it was reported that this milling approach could remove layers as thin as 15 nm from the blockface, and following unpublished experiments have revealed that this value can be even decreased to few nanometers. This difference in thickness between FIBSEM and SBEM is probably due to changes in the resin structure due to imaging which differentially affects the two cutting mechanisms. To image the tissue at a resolution high enough to see synapses, significant energy must be delivered to the block-face, resulting in changes in the structure of the resin (e.g. hardening due to crosslinking) which make the sectioning with the diamond knife difficult, but do not represent a problem for the ion beam (Knott et al., 2008). The current major limitation of FIBSEM is the size of the area that can be milled with the ion beam, because the focused ion beam cannot be deflected arbitrarily. FIBSEM is therefore less suited for imaging of large volumes.

FIBSEM has been shown to be a powerful technique to identify, reconstruct and quantify asymmetric and symmetric synapses from large three-dimensional tissue samples, with the ability to classify virtually all synaptic junctions regardless of the plane of sectioning thanks to the high z-resolution and the natural alignment of the sections due to imaging of the blockface (Merchan-Perez et al., 2009; Morales et al., 2011). Merchan-Pérez and coworkers imaged mouse brain tissue with a FIBSEM with a later resolution of around 4 nm and a milling thickness of around 20 nm. First, they observed that regarding ultrastructural detail the acquired images were comparable to TEM micrographs, with no damage caused to the ultrastructure by the ion beam. They then reconstructed synapses and noted that even when a synaptic junction was sectioned en face, it was still possible to estimate the thickness of the synaptic densities and to digitally reslice the stack of consecutive images through a perpendicular plane of section, allowing to classify the synapse as symmetric (inhibitory) or asymmetric (excitatory). This last point is very useful for stereology to count synapses in a volume, because stereological approaches are limited in estimating the proportion of symmetric versus asymmetric synapses, since a high percentage of the synapses (40-60%) cannot be characterized from the analysis of single sections due to the orientation of the synaptic junction parallel to the sectioning plane (DeFelipe et al., 1999). In fact, a framework and software to automatically segment and count
synapses in FIBSEM data, called ESPINA, has been introduced (Morales et al., 2011).

1.3 Limitation of EM-based methods for studying neural projections

The methods introduced in the previous section are mainly based on electron microscopy. Although this results in high resolution that allows unambiguous classification of synapses, these methods suffer from the inherent drawback of electron microscopy of necessitating long imaging and tracing time, in the order of years for volumes close to 1 mm$^3$ (see (Helmstaedter, Briggman, and Denk, 2008) for estimations of reconstruction time). The consequence of this is that complete EM reconstructions of vertebrate brains require prohibitive amount of time and are thus not feasible with current technology. For many biological questions related to neural connectivity, however, it is of high interest and necessary to identify the neurons inside a reconstructed volume. Neurons in an area of interest can come from long-range projections originating several millimeters away, and are therefore not contained in their entirety in the reconstructed volume. Since imaging and tracing time are too high to trace these axons back to their soma (in addition to the problem of finding a way to embed and image an entire brain as a single piece), it is therefore necessary to mark them so that their origin can be determined based on a label that they contain and which is directly visible in the EM, or can be deduced by correlating the EM data with other images.

We have discussed in a previous section how electron dense tracers have been used to investigate neural connectivity (see 1.2.1). BDA or HRP, for example, can be injected in one brain area, either intracellular or as a bulk injection, diffuse in the neurons, and can be detected in the EM in the region of interest, several millimeters away (Anderson et al., 1994; da Costa and Martin, 2009). Electron-dense tracers have however the disadvantage that only a limited number of them can be used simultaneously. Two different electron-dense tracers have been combined many times (Smith and Bolam, 1991), but only few studies have successfully used three different electron-dense markers simultaneously (Reiner et al., 2000). An example of triple labeling was done by Anderson and coworkers using immunohistochemistry to study multiple inputs to tegmental dopaminergic neurons (Anderson, Karle, and Reiner, 1994). The authors investigated the role of two types of striatal neurons projecting to the tegmentum, one characterized by the expression of substance P (SP), the other of preproenkephalin-derived neuropeptides (ENK), and in particular
addressed the question of whether synaptic terminals from the two types of inputs segregate on separate populations of tegmental dopaminergic and non-dopaminergic neurons. To answer this question, they developed a method which combines three markers which can be distinguished at both the LM and EM level, diaminobenzidine (DAB), benzidine dihydrochloride (BDHC) and silver-intensified immunogold (SIG). In the LM and prior to osmification, the three markers had different color and distribution, brown and diffuse in the case of DAB, blue and crystalline for BDHC, black, dense and punctate for SIG. After osmification and in the EM, the three markers were distinct in their structure, DAB formed a black and flocculent precipitate which filled the terminals, BDHC labeling consisted of discrete clumps of crystalline and elongate reaction product with a dark flocculent background (in many cases the clumps extended outside the cell boundaries), and the SIG labeling appeared as black, nearly round particles (Fig. 1.3). By using the three markers to label SP, ENK and tyrosine hydroxylase (TH), the authors were able to show that although SP-positive and ENK-positive terminals simultaneously contacted TH-positive dendrites in the ventral tegmental area, they most commonly converged on non-dopaminergic dendrites. It should be noted that this pre-embedding procedure has some drawbacks: it requires a long pre-embedding histochemical procedure, causes degradation in the tissue ultrastructure, and leads to a substrate which, in the case of one marker, is not confined to the labeled structure.

Figure 1.3: Example of tracing with three electron-dense markers used simultaneously. An SP-positive terminal, labeled with DAB, and an ENK-positive terminal, labeled with SIG, form appositions (arrows) with a dopaminergic neuron, labeled with BDHC. Scale bar: 500 nm. Image adapted from (Anderson et al., 1994).
Procedures to detect multiple markers have also been applied to detect multiple tracers labeling axons in the same section. Lanciego et al. introduced a triple labeling combining retrograde labeling with PHA-L, BDA and retrograde labeling with Fluoro-Gold and tested it to study convergence of different sets of projections within the thalamus (Lanciego et al., 1998). The protocol combined injection of the tracers and their detection using antibodies and different substrates, nickel-enhanced diaminobenzidine (DAB-Ni), DAB, and Vector VIP, a peroxidase substrate. The staining resulted in precipitates of different colors, black, brown, and purple, which could be easily distinguished in the LM. Unfortunately, although the precipitates were electron-dense, the authors did not prepare the section for electron microscopy, only suggesting that a method based on the approach was attractive for testing for EM.

The absence of multiple electron-dense markers (more than 2-3) that can be used simultaneously to identify different neuron populations, results in a potential difficulty of describing connectivity between different brain regions in terms of interactions between different projection neuron types. The premotor nucleus HVC of zebra finch, for example, projects to at least 6 different areas of the brain (Foster and Bottjer, 1998; Akutagawa and Konishi, 2010), and it would be therefore impossible with current EM-labeling techniques to label all these populations with distinguishable electron-dense markers. LM, and in particular fluorescence LM, can overcome this limitation of few distinguishable electron-dense tracers and allows to simultaneously label multiple cell population with distinct markers.

1.4 Light microscopy based reconstruction methods

Starting from the 1960s, morphology and connectivity of neurons started to be investigated with a new class of markers based on fluorescent dyes (Callahan, Yoshikawa, and Thomas, 1998). Fluorescent tracers have the advantage of allowing to trace multiple pathways at the same time, by combining fluorophores which are excited and emit light at different wavelengths. In 1968, Stretton and Kravitz electrophoretically injected the fluorescent dye Procion Yellow into identified cell bodies of the lobster abdominal ganglia (Stretton and Kravitz, 1968). The dye spread into the fine branches of the cells, survived fixation and histological preparation, and remained in place, allowing to reconstruct the cell shape by examination of serial sections of the ganglia. By repeating the injection of the same cell in different animals, the authors observed that cells have characteristic shapes and that the neuropil of the ganglia is highly structured.
Ten years later, a much more sensitive dye was introduced to study functional connections between cells, Lucifer Yellow (Stewart, 1978). Stewart injected this dye and Procion Yellow into neurons of a mollusk ganglia, finding much stronger fluorescence and higher amount of fine, complex branching. Moreover, he observed low toxicity of the dye, fast diffusion, and unchanged distribution of the dye by fixation and embedding.

Importantly, fluorescent dyes have not only been used as intracellular dyes, but, starting with attempts at retrograde labeling with Evans Blue dye combined with cattle albumin (Köbbert et al., 2000), have also been introduced for tract-tracing, both as anterograde and retrograde tracers. A large number of molecules can currently be used as fluorescent tracers, including Nuclear Yellow and Fast Blue (Bentivoglio et al., 1980), Fluoro-Gold (Persson and Havton, 2009), fluorescently tagged cholera toxin subunit B, a highly sensitive retrograde tracer (Conte, Kamishina, and Reep, 2009), and dextran amines (Glover, Petursdottir, and Jansen, 1986).

Dextran amines (which we already mentioned in section 1.2.1 in the context of BDA) are hydrophilic polysaccharides characterized by good water solubility and low toxicity. Thanks to an uncommon poly-(α-D-1,6-glucose) linkage, they are resistant to cleavage by most endogenous cellular glycosidases and are biologically inert (Johnson and Spence, 2011). Dextrans can differ from each other in their molecular weight, from moderate (3000 DA) to high (40000 DA or more), and this affects the transport rate and filling of peripheral processes. Fritzsch measured the speed of anterograde and retrograde axonal movement for dextran amines of 3000, 10000 and 40000 molecular weight conjugated to different fluorochromes (Fritzsch, 1993). To compare the distance traveled by the different tracers in the same amount of time, he applied them to the cut lateral nerve of *Xenopus laevis*, which run for several centimeters straight along the body wall. For all tracers, except for the 40 kDa, he observed both anterograde and retrograde movement. In particular, 3 kDa dextran amines moved 2 mm/h, twice as fast as 10 kDa dextran amines (1 mm/h, both were measured at 22°C), and the speed was independent of the fluorochrome (he tested Cascade blue, Fluorescein, Texas Red and rhodamine). Moreover, he observed in the LM that 3 kDa dextran amines filled fine profiles not detectable with 10 kDa dextrans. Nance and Burns investigated the difference of five different conjugated dextrans (Lucifer Yellow, Texas Red, Fluorescein, Cascade blue and Tetramethylrhodamine) of molecular weight 10000 in anterograde tracing (Nance and Burns, 1990). They injected the dextran amine by pressure injection into the brain, dorsal root ganglia or foot pads of adult rats, and, after a survival time of 3 to 14 days, they perfused the animals, prepared sections and imaged them. In their case, possibly due to the injection type and the different animal system,
they found difference in labeling by the different tracers, with intense labeling of cell bodies, axons and dendritic processes at the injection site by Texas Red, Fluorescein and Tetramethylrhodamine and predominant but not exclusive anterograde labeling, and less staining for the other tracers.

Fluorescence can be used to trace neurons not only by injecting fluorophores, but also by using viral or genetic tools to let the cells express fluorescent proteins such as green fluorescent protein and its variants. Several recombinant viral vector can be used for long-term expression of fluorophores without significant toxicity to the neurons, including for example adeno-associated virus (AAV) and lentiviruses derived by HIV (Luo, Callaway, and Svoboda, 2008). Additionally, some vectors allows to establish synaptic connectivity by their ability to cross synapses. We will describe experiments we have conducted with AAV vectors in the songbird in chapter 4.

**Brainbow**

A recent method of fluorescence labeling to study neural connectivity is the Brainbow (Livet et al., 2007). In this method, synaptic circuits are visualized by genetically labeling neurons with combination of multiple and distinct colors. The Brainbow is based on the Cre/lox recombination system, which allows switching gene expression by DNA excision, inversion, or interchromosomal recombination. Transgenic animals have a construct containing several fluorescent proteins, such as green, red and yellow fluorescent protein, alternated by lox sites. Brainbow transgenes drive the combinatorial expression of these proteins, by using Cre to mediate either excision between pairs of incompatible lox sites, alternated to create mutually exclusive recombination events, or inversion of DNA segments positioned in tandem to generate several recombination outcomes (Weissman et al., 2011). As a result, multiple copies of the construct within the same cell express different combination of proteins, generating fluorescent protein mixtures that allow to label individual neurons with as many as 90 distinguishable colors. In other words, whereas combinatorial expression of three different fluorescent proteins can color neurons in one of ten hues, the expression of these fluorescent proteins in different amounts in different neurons lead to a much larger number of hues (Lichtman, Livet, and Sanes, 2008). The authors made an example of the utility of the Brainbow approach by illustrating the example of the innervation of skeletal muscles by motor axons. If one wanted to investigate the connectional maps made by the motor axons on a single muscle, with conventional fluorescence tracing methods (e.g. expression of GFP in the motoneurons) it was necessary to acquire a high number of high-
resolution and overlapping confocal stack to cover the whole muscle and to be able to trace a single motor axon over the entire muscle. With Brainbow, the authors claim that the process of reconstructing the 'connectome' of a muscle can be accomplished in much less time, because in some cases it is possible to identify the same axons at multiple position without having to trace its path, because its unique color remains recognizable along its length (Lichtman, Livet, and Sanes, 2008).

Interestingly, the Brainbow approach has been recently ported to the Drosophila in two systems, the Drosophila Brainbow (dBrainbow) and the Flybow (Hadjieconomou et al., 2011; Cachero and Jefferis, 2011; Hampel et al., 2011). A unique feature of the Drosophila Brainbow systems and an improvement over the original Brainbow, is the control of which neurons are labeled using the Gal4-upstream activating sequence expression system. In fact thousands of fly Drosophila lines are available which express Gal4 in different cell populations, allowing targeting the Brainbow to identified neurons. The Drosophila Brainbow systems can be therefore used for example to identify neuron subtypes within a neuron population, or to facilitate tracing of individual neurons in a neural circuit. Moreover, the expression of Brainbow construct could be in principle driven only in cells expressing specific neurotransmitters, or by transcription factors linked to specific behaviors, thus helping to related these behaviors to specific groups of neurons.

The Brainbow has however the limitation that the colors of different neurons are not independent and thus a neural process can not be unambiguously identified if its diameter is below LM resolution, as it can be the case for spines and nonmyelinated axons (Helmstaedter, Briggman, and Denk, 2008). Moreover, because of the same resolution limitation, synapses must be stained and can also not be unambiguously identified, a problem common to all LM-based methods.

**Superresolution methods**

Recent advances in LM development have increased LM resolution beyond Abbé’s diffraction limit. The most prominent example is stimulated emission depletion (STED) microscopy (Hell and Wichmann, 1994). STED is based on the principle that the size of the fluorescence spot in a scanning fluorescence microscope can be decreased by stimulating with a second laser beam the fluorescent emission in its outer region. This second doughnut-shaped laser beam, called the STED beam, has the effect of de-exciting the fluorescent molecules by stimulated emission at a wavelength longer than the fluo-
rescence, and thus of switching off the fluorescence in the outer region and of sharpening the shape of the fluorescence spot (Nägerl and Bonhoeffer, 2010; Leung and Chou, 2011). Applied to the imaging of hippocampal neurons, STED has been shown to allow to reach resolution better than 100 nm and thus to visualize details including spine neck width and the curvature of the heads of spines (Nägerl et al., 2008).

In addition to STED, in recent years other super-resolution imaging techniques have been developed, some based on the principle that the position of a spatially isolated fluorophore can be resolved with a precision higher than its point spread function. These techniques include stochastic optical reconstruction microscopy (STORM) and photoactivated localization microscopy (PALM) (Leung and Chou, 2011).

Despite the increase in resolution achieved by these super-resolution techniques, they still do not allow to detect exhaustively small details such as synaptic vesicles and synaptic membrane specializations in a tissue volume, and they cannot therefore replace EM.

**Array tomography**

A method which has been advertised for circuit reconstruction in recent years and is based on light microscopy, but is compatible with electron microscopy, is array tomography, a proteomic imaging method which allows for quantitative, large-field volumetric imaging of biological tissue (Micheva and Smith, 2007).

The technique is based on the idea of improving volumetric resolution of immunofluorescence microscopy by working on its major limitation, the resolution along the z-axis. In contrast to optical sectioning done with confocal microscopy, which can reach a resolution of around 700 nm at best, the tissue is physically sectioned in ultrathin sections, thus bringing the z-resolution to the same value as the section thickness.

The advantage of using ultrathin sections for z-resolution was already noted by Mori et al. studying the subcellular distribution of different antigens in human placental endothelial cells, who observed that the z-resolution improvement achieved by immunofluorescence microscopy on ultrathin sections allowed him to resolve the precise subcellular distribution of the antigens and to analyze quantitatively much better than conventional or confocal light microscopy (Mori et al., 2006). However, whereas this last study used cryosections, array tomography was proposed to be done with the acrylic resin LR White, which has the advantage of being much easier to handle than cryosections, is said to be better than other resins for immunostaining thanks to its hydrophilic properties, and can preserve GFP fluorescence. Luby-Phelps and coworkers looked
at photoreceptors in the zebrafish retina overexpressing GFP in semithin sections (Luby-Phelps et al., 2003). These were prepared by fixation in 4% paraformaldehyde/0.1% glutaraldehyde and embedded without further staining in LR-White. GFP fluorescence was still visible after embedding, and also its antigenicity was retained, allowing the researchers to perform gold anti-GFP immunostaining to detect GFP in the EM.

In array tomography, the embedded tissue is cut in ribbons of serial ultrathin sections, which are collected on a single coated glass slide. Sections encompassing substantial tissue volumes can therefore be simultaneously processed, for example by immunostaining them, and imaged, allowing the researcher to reconstruct the three-dimensional distribution of antigens. Micheva and Smith introduced array tomography by testing it on adult mouse cerebral cortex. They first compared resolution of tissue prepared with array tomography with tissue imaged with a confocal microscope, in both cases by looking at immunostaining of common antigens, synapsin and α-tubulin. Although single confocal optical sections had more signal, summation of multiple ultrathin sections covering the same volume revealed that the amount of signal was comparable, with the advantage of ultrathin section of revealing small immunolabeled structures, which were lost in the confocal images due to the brighter and bigger immunolabeled points, and most importantly to have a much higher z-resolution. The increase in resolution allowed discriminating individual objects which in confocal microscopy were not resolvable. Moreover, immunostaining on ultrathin sections allowed achieving uniform staining through the depth of the tissue, something which is difficult to achieve with immunostaining of vibratome sections due to limited penetration of the antibodies even when using detergents. Micheva and Smith also noted that fluorescence of the antibodies showed less bleaching than when applied to vibratome sections, and that a fluorescent protein expressed in the tissue, YFP, was preserved after embedding and sectioning, allowing to investigate the distribution of antigens relative to identified cells.

An important approach introduced with array tomography is consecutive staining with antibody elution. Antibody elution was a technique explored in the past to overcome some of the limitations of immunostaining, in particular the fact that for many years only few fluorophores were available that could be used simultaneously, and the fact that most antibodies originate from a couple of animal species only, mainly rabbit and mouse, thus limiting the number of combinations of primary antibodies (Tramu, Pillez, and Leonardelli, 1978; Wählby et al., 2002). Using an acidic solution, it was shown that it was possible to elute the first applied primary and secondary (in this case peroxidase-labeled) antibodies, to then perform an additional staining with another antibody, which could be raised in the same species as the one in the first round (Nakane, 1968).
Alternatively, the cross-reactivity of primary antibody could be blocked by heating of the sample, which also has the advantage of increased cytoplasmic and nuclear antigen detection (Lan et al., 1995). Finally, it was also shown that a combination of elution by low pH solution and denaturation by heating was feasible for sequential immunofluorescence staining with up to three rounds of staining (Wählby et al., 2002).

Micheva and Smith applied the antibody elution approach to the LR White sections they prepared, showing that multiple consecutive rounds of staining could be performed (they showed nine rounds). They imaged the sections after each immunostaining round, and used an antibody of the first staining which was included in all successive round to align the images. Thanks to the fact that the sections were embedded in a resin, damage of the tissue was limited, and they observed no apparent decrease in antigenicity due to the chemicals applied. The authors noted that by using multiple antibodies during each cycle, for example 4, it was possible using array tomography to localize with 9 elution cycles 36 different antigens, thus helping visualize the molecular complexity of the tissue. Micheva et al. applied array tomography to determine the composition of glutamate and GABA synapses in the somatosensory cortex of YFP transgenic mice (Micheva et al., 2010). The authors noted that within each neurotransmitter synapse category, there is substantial diversity in the synaptic proteins (e.g. neurotransmitter transporters and receptors) expressed, and thus investigation of the molecular diversity of synapses may help understand their development and physiology. In addition, they observed that a systematic understanding of synapse diversity (which they called "synaptome") could also provide information about synaptic circuitry, and that array tomography, with its ability to visualize multiple antigens in the same tissue, has the potential to describe molecular diversity with fast imaging of large volumes of tissue.

The authors used up to 17 markers to identify synapses (e.g. synaptophysin, bassoon, and synapsin) and started to classify them based on the presence of specific markers. For example, based on the presence of vesicular glutamate transporters, they could divide glutamatergic synapses in different groups (containing either one of two transporters, both or none), and to quantify these in different layers. Moreover, thanks to the speed of classic light microscopy, they were able to achieve a high experimental throughput, with a claimed semi-automatic acquisition of one million synaptic protein puncta per hour.

An intriguing potential of array tomography is the ability to look at the ultra-thin sections in both the LM and EM. Micheva and Smith used the sections mounted on glass slides and imaged them with an SEM. It should be noted, however, that the sections were prepared differently than those used for light microscopic imaging only. In particular they added postfixation in OsO₄, which
is necessary to preserve ultrastructural details, with the disadvantage of limiting the number of antibodies that could be used. They labeled the sections with antibodies against GABA and tubulin, imaged the sections, eluted the antibodies, restained with β-actin and SNAP-25 antibodies, imaged the sections, and then, after poststaining, relocated the region in the SEM (using landmarks visible in both imaging modes, such as blood vessels and cell nuclei) and imaged it (Micheva and Smith, 2007). After alignment of the images, they were able to locate immunofluorescent signal relative to features visible at the EM, for example tubulin was observed on microtubules, consistent with the literature, and GABA confined to presynaptic boutons and axonal varicosities. Similarly, in their study about molecular synaptic architecture, they analyzed distribution of synapsin immunostaining, which they wanted to use as a general synaptic marker, by performing immunostaining on osmified sections, and found that it stained 91% of the synapses (i.e. 91% of the synapses were synapsin positive in at least one section; it should be noted however that this analysis does not consider false positives). This preparation allows to localize the antigen relative to ultrastructural features, however it results in a tissue quality which is in our opinion not sufficient for a reliable detection of synapses, nor for tracing of neurons (Fig. 1.4).

Correlation of EM and LM data offers in principle a great advantage, namely the combination of multicolor, large-scale information with high-resolution ultrastructure data. In the present thesis, we explored the potential of array tomography as a correlative tool to study neural connectivity. In particular, we based our strategy on the same idea of imaging arrays of serial section in both LM and EM, but changed different parts of the protocol in order to achieve
better EM quality. First, however, we would like to give an overview of how correlative microscopy has been used to study neurons and networks of neurons.

1.5 Correlative light- and electron microscopy methods

Several correlative light and electron microscopy (CLEM) approaches have been used in the past and are currently developed. We will distinguish here two main groups, those based on correlation of transmitted LM with EM, such as the ones using electron-dense tracers imaged in both microscopes, and those on fluorescence LM and EM, such as correlative array tomography.

1.5.1 Transmitted LM with EM

Correlative transmitted light and electron microscopy methods have been used since the 1960s, with first attempts to image neurons stained with the Golgi method, a technique developed for light microscopy which allowed the researchers to visualize neurons in their entirety in the electron microscope (Blackstad, 1965). These firsts experiments suffered from problems related to difficulty of ultrathin sectioning, section instability, and obscuration of the ultrastructure due to the precipitate, but the technique was later improved by developing deimpregnation methods that only left a much more localized label in the neurons (Fairen, 2005). Later on, as we have discussed in section 1.2.1, more methods were developed, including HRP and BDA, which are both visible in the LM and EM. In addition to these methods, immunogold has also been used for CLEM.

1.5.2 Fluorescence LM with EM with correlative markers

Whereas correlative transmitted LM and EM only allows to distinguish few different labels, correlation of fluorescence microscopy with electron microscopy has the advantage in principle of allowing many more labels to be correlated and, in addition, has been used several times with genetically encoded markers, such as GFP or activity indicators.

Photooxidation

A widely used method that allows correlating fluorescence microscopy with EM is conversion of the fluorescent marker into an electron-dense marker which is both visible in the LM and EM. The method takes advantage of the fact that fluorophores when illuminated release oxygen radicals which can be used to
oxidize DAB added to the tissue, generating an electron-dense precipitate localized where the fluorescence was. Photooxidation has been developed in 1982 by Maranto who examined neurons in the leech and crayfish (Maranto, 1982). He injected into neurons Lucifer Yellow, a marker which at that time was more easily injected and more sensitive at the level of light microscopy than other dyes. After irradiation with intense blue light in the presence of DAB for 30 minutes, a reddish-brown reaction product was visible within the injected cells. He then processed the section for electron microscopy (osmification and embedding in resin) and found the precipitate to be visible even in small processes. Inspection of the ultrathin section in the EM revealed that the precipitate was well visible, but not obscuring synaptic vesicles.

Photooxidation has the advantage of allowing to reach very high resolution. Deerinck and coworkers used eosin, a fluorescent molecule with a particularly high singlet oxygen quantum yield, conjugated to antibodies which they used as secondary antibodies to immunolabel microtubules (Deerinck et al., 1994). Through photooxidation of eosin, they were able to delineate in electron micrographs individual stained microtubules, with very fine granular structure and minimal diffusion of the reaction product under optimal conditions. Another advantage of photooxidation is that a wide range of fluorophores can be used, including tracers such as Fluorogold or Tetramethylrhodamine conjugated to dextrans, red fluorescent latex microspheres and carbocyanine dyes (for a review see (Modla and Czymmek, 2011)).

A major disadvantage of photooxidation is the limited volume of tissue which can be processed. Photooxidation requires strong illumination of the tissue containing the fluorophores, which is normally achieved by shining light to the probe through an high-aperture microscope lens, limiting the reaction to the tissue in the field of view of the lens (which tends to be relatively small with high-aperture lenses). Also, photooxidation tends to be non uniform along the z-axis, with less staining deep in the tissue. Another disadvantage of photooxidation, which however can be chemically or temporally (by monitoring the reaction) controlled, is that unspecific staining occurs if autofluorescence is present in the tissue, thus causing conversion of DAB at unspecific positions.

Recently a new type a marker which can be used for photoconversion has been developed, miniSOG, which has the advantage of being genetically encoded.

miniSOG

MiniSOG is a genetically encoded tag based on a fluorescent flavoprotein engineered from Arabidopsis phototropin 2 (Shu et al., 2011). MiniSOG is a small protein, containing only 106 amino acids (less than half the size of GFP), and
its illumination leads to generation of sufficient singlet oxygen to catalyze photooxidation of DAB. MiniSOG emits green fluorescence, with an intensity lower than GFP but still enough to be easily detected, as shown by the authors who tagged miniSOG to different proteins and localized it for example in different cytosolic compartments, in the secretory pathway, in mitochondria and in nuclear histones. MiniSOG was also fused to SynCAM1 and SynCAM2, to study their synaptic distribution, which was previously unclear. After photooxidation, the authors examined the distribution at the EM, and found SynCAM1-miniSOG only at presynaptic terminals, and SynCAM2 at postsynaptic sites. Interestingly, the authors also inserted these fusion proteins into prenatal mouse brains by \textit{in utero} electroporation and imaged these with SBEM. Thanks to the strong osmiophilicity of the reaction product of the photooxidation and the combination with \textit{en bloc} uranyl acetate staining, the staining at the synapses was well visible as backscattered electron signal in the SEM was strong. In this way, it is in principle possible to combine large-scale three-dimensional reconstruction of tissue with detection of specific synapses genetically tagged.

MiniSOG is not the first genetically encoded EM marker. Transgenic enzyme markers have been previously developed, in particular HRP cDNA markers which could be placed under the control of different promoters (e.g. synapsin for neuron-specific expression) or could be flanked by specific sequences (e.g. endoplasmic reticulum retention signal for expression localized within the EM (Schikorski, Young, and Hu, 2007)). (Li et al., 2010) developed a membrane targeted HRP (mHRP) construct which was coexpressed with EGFP to label the full extent of a neural structure, with uniform labeling of the whole neuron and no effect on development of dendritic arbors. This construct has been proposed by the authors to be ideal for combining \textit{in vivo} time-lapse imaging with serial section EM of the same transfected neurons, with the mHRP expression as an help to reconstruct neurons, in particular when they are very small and difficult to trace.

Although this last approach allows correlating LM and EM, MiniSOG represents a truly correlative marker, because it is the fluorescent protein itself that catalyzes (via single oxygen) the production of a highly localized substrate. The mHRP-EGFP construct, in contrast, leads to the expression of two proteins which label the same neurons, but not the same subcellular structure (mHRP is localized to membranes). Moreover, thanks to the small size of miniSOG, it can be directly tagged to different proteins, therefore allowing to localize their position with high precision.
Quantum dots

Quantum dots are a correlative marker visible in both LM and EM (Giepmans et al., 2005). They are semiconductor nanocrystals, typically with a core consisting of cadmium selenide and a zinc sulfide shell, and their tightly packed atoms make them electron-dense. Importantly, quantum dots are also fluorescent, and quantum dots of different size have distinct emission peaks. Giepmans et al. investigated their use as pre-embedding markers for CLEM. They showed that quantum dots can be used for double and triple labeling of proteins, with the quantum dots distinguishable at the LM levels thanks to different emission spectra, and at the EM levels thanks to the different shapes and sizes different quantum dots have.

Unfortunately, quantum dots have some disadvantages (Modla and Czymmek, 2011). First, when used preembedding their penetration is limited to few micrometers, thus limiting their potential for pre-embedding labeling of large tissue volumes. Secondly, the electron density of quantum dots is relatively low and these are therefore difficult to distinguish from the tissue background. This low visibility requires high magnification to locate them (and also to distinguish different shapes), thus affecting imaging time in the EM. On the other side, however, development of quantum dots in a form that can be used as neural tracer, for example coupled to dextran, would be of high interest to have distinguishable marker which are visible in both LM and EM.

1.5.3 Fluorescence LM with EM through overlay

The correlative methods described in the previous sections are based on the presence of markers in the structures of interest visible with both LM and EM. An alternative approach is to overlay images acquired with the two microscopes without the structures of interest being labeled in both microscopes, but using features visible in both microscopes to align the images. An example of this approach is correlative array tomography, which we will explore in the present thesis.

In some cases, the motivation of overlay techniques is to avoid some of the compromises that are present when having markers visible in both LM and EM, for example the ultrastructure decrease due to permeabilization in preembedding immunolabeling or the limited tissue volume that can be processed with photooxidation, and to gain the advantage of being able to use many more markers. In other cases, overlay techniques are used to combine in vivo imaging with electron microscopy of the same tissue. In principle, the ideal case would be to acquire images with any fluorescent marker in the LM, then prepare the tissue for electron microscopy with the best possible ultrastructure quality, and then
correlate two volumes with informatics tools that allow to find corresponding structures and correct for transformations in the tissue. Today this is unfortunately still beyond the power of the available tools, limiting overlay approaches to direct correlation of big structures only (e.g. somata) or needing some intermediate steps to find corresponding features.

**Correlation pre-embedding to EM using endogenous features**

An example of direct correlation of LM with EM data has been presented in section 1.2.3. Briggman et al. correlated two-photon calcium imaging with the same tissue volume acquired with a SBEM. To this purpose, they used vasculature landmarks, and were able to relocate the cell bodies of the cells previously imaged with two-photon imaging (Briggman, Helmstaedter, and Denk, 2011). Similarly, Bock et al. also used vasculature for their alignment of *in vivo* images with ssTEM data of the same volume, in particular using blood vessels of successively finer calibre to identify individual neurons (Bock et al., 2011).

**Near-infrared branding**

An interesting approach to correlate small fluorescently-labeled (e.g. by GFP) structures such as dendrites is to insert in the tissue fiducial marks visible in both LM and EM. Near-infrared lasers have been used to create three-dimensional reference marks that are fluorescent in the LM and can be photooxidized to generate electron contrast (Bishop et al., 2011). In near-infrared branding (NIRB), a femtosecond pulsed titanium-sapphire laser is used to create user-defined pattern of NIRB marks, the size of which can be controlled by the dose of pulsed light and the position of which can be determined with micrometer precision to be close to a structure of interest. These marks are autofluorescent and, thanks to their spectral features, can be photooxidized without driving photooxidation of GFP. In the EM, the marks can be recognized as tissue defects surrounded by electron-dense material and used later to register electron with light micrographs. Bishop et al. showed how individual spines of apical dendrites of fluorescently labeled cortical neurons could be reidentified in the EM by burning a box of 6.5 μm x 6.5 μm around the spine, and an additional 25 μm x 25 μm box to help trimming. This last box was important for preparation, because the embedded block did not contain any other marker visible at the LM (the GFP fluorescence was not visible anymore) and therefore the box was helpful to trim the block at the correct position. It should be noted that although this approach is very promising to targeted detection of individual structures, it is probably less appropriate for imaging of volumes with high density of structures, because
it destroys the marked tissue.

High-pressure freezing

High-pressure freezing techniques are used for correlative microscopy, in particular when the preservation of the exact state of a living cell is particularly important. Living cells can be observed by LM and high-pressure frozen within few seconds (4-5 seconds at best) to capture a biological event of interest. The same sample can then be imaged in the EM and correlated with light micrographs. This correlative approach, as noted in (McDonald, 2009), has several advantages over high-pressure freezing alone and over classical chemical preparation. First, as in other correlative approaches, LM provides contextual information, but also information about how the cells behaved shortly before fixation. Second, a dynamic process can be imaged at high resolution at a known point tracked by light microscopy. Third, LM allows locating a rare event, both temporally and spatially.

Sims and Hardin used this approach to investigate *C. elegans* embryos expressing GFP depending on their genotype (Sims and Hardin, 2007). The fluorescent tag allowed them to select only specific embryos for TEM observation. These were embedded in agarose and observed with a LM and, after high-pressure freezing, were relocated in a TEM. The LM and EM were overlaid so that they could identify which organelles were giving rise to the fluorescence signal. It is interesting to note that, in addition to preembedding light micrographs, they were able to image postembedding GFP fluorescence in plastic ultrathin sections.

An example of the high temporal resolution which can be achieved with correlative microscopy with high-pressure freezing is shown by the Rapid Transfer System developed by P. Verkade (Verkade, 2008), which allows to fix a specimen in less than 5 seconds, and was used to investigate endomembrane transport events. To help in the correlation, before embedding the sample is placed on a grid, which can be used as a reference. After embedding, the grid can be removed but it remains visible in the morphology of the resin block, allowing to relocate the quadrant containing the cells of interest.

High-pressure freezing is however not well suited for neural circuit reconstruction, because the size of the tissue which can be processed is very small and, on the other side, large brains are difficult to dissect in short time if they have not been fixed.
Direct correlation

Correlation of LM and EM has also been done on ultrathin cryosections. In this case, similar to correlative array tomography, the overlay is between images of the same section, and is therefore much easier than the previous methods, because no transformation (or only minimal) of the section occurs between the two imaging moments (Robinson et al., 2001).

Pombo et al. imaged transcription sites in human cell nuclei using immunostaining on ultrathin cryosections (Pombo, Hollinshead, and Cook, 1999). The cells were first permeabilized and then incubated with Br-UTP, which incorporated into RNA. Using antibodies against Br-RNA followed by secondary antibodies conjugated to fluorophores and gold particles, nascent transcripts at the site of transcription were labeled after embedding and cryosectioning. The antibodies could be detected both in the LM, thanks to the fluorophores, and in the EM, thanks to the gold particles. Similarly, Takizawa et al. used FluoroNanogold, a 1.4-nm gold cluster compound to which antibodies are conjugated, to detect molecules such as lactoferrin (a marker protein found in human neutrophils) in both fluorescence and electron microscopy (Takizawa, Suzuki, and Robinson, 1998).

1.6 Correlative array tomography

Array tomography, as introduced in section 1.4, can be used as an overlay correlative technique. The same ultrathin sections, collected on glass, can be imaged in both LM and EM, thus allowing for combination of fluorescence signal of antibodies used to detect various antigens with ultrastructure information of the same tissue region after alignment of the images acquired with the two microscopes. In the next chapter, we will illustrate how we implemented correlative array tomography in the songbird system. We adapted correlative array tomography for the study of projection neurons between different vocal brain areas in the zebra finch, by injecting tracers of different colors into different areas and using their fluorescence or detection with antibodies in ultrathin sections to classify structures imaged in the EM, allowing for classification of neurons and their synapses.
Chapter 2

Correlative array tomography to investigate projection neurons’ connectivity

2.1 Introduction

In the project presented in this thesis, we decided to adapt correlative array tomography for the study of neural connections in the songbird. We decided to take advantage of the fact that most areas in the songbird song system are segregated in discrete nuclei, and therefore different nuclei can be targeted with injections of neural tracers of different colors. In this way, neurons belonging to different populations (such as neurons in HVC, in which several populations coexist) can be distinguished based on their fluorescent label. At the same time, we decided to use electron microscopy to detect synapses because it allows us to unambiguously identify them. The main problem of combining fluorescence LM and EM is that tissue preparation for the two microscopy modalities is different and tends to be incompatible. Whereas electron microscopy often requires strong fixation and staining of the tissue, fluorescence dyes tend to be quenched if the tissue is prepared for EM. This incompatibility results in either good ultrastructure but loss of fluorescence, or good fluorescence but low ultrastructure quality.

We developed a preparation protocol based on array tomography that overcomes some of the problems of incompatibility in tissue preparation. Our approach allows to combine multicolor fluorescence information with synapse identification and neuron classification in the electron microscope. We inject tracers of different colors into the living brain and, after the tracers have diffused, we perfuse...
the animal. After vibratome sectioning, we process the sections for EM. The tissue is stained and fixed with heavy metals, dehydrated in a graded series of ethanol dilutions, and finally embedded in an epoxy resin. We cut the tissue in ribbons of ultrathin serial sections and image the collected ultrathin sections in a conventional wide-field fluorescence microscope. Preparation of the tissue for EM causes a strong reduction of the tracers fluorescence, and we recover tracer signal by immunolabeling against the fluorophores. We finally image the sections in the EM, and align them with LM images using various landmarks. The resulting data consists of multichannel LM pictures superimposed on EM imagery.

In this chapter we will illustrate the various preparation step and the resulting data (section 2.2), discuss the results, the protocol and compare it to other methods (section 2.3), and provide a detailed preparation protocol (section 2.4).

2.2 Results

2.2.1 In vivo fluorescence labeling

Our correlative approach is based on combining fluorescence light microscopy and electron microscopy. Fluorescence light microscopy provides information about neurons and their synapses inside a small subvolume, which is then imaged in the electron microscope. Fluorescence information is provided by neural tracers, which are injected \textit{in vivo} into different regions of the songbird brain. We mainly used dextrans of 10000 MW, conjugated to different fluorophores such as Alexa Fluor dyes, Tetramethylrhodamine (Fluoro-Ruby), Texas Red and Lucifer Yellow. The tracers are pressure injected \textit{in vivo} into different brain areas during a surgical procedure in which the animal is fixated in a stereotaxic apparatus. Fig. 2.1 is an example of retrograde tracing in the nucleus LMAN after injection of Fluoro-Ruby dextran in RA. The tracer diffused from the axonal terminals in RA back to the somata in LMAN, so that the nucleus was clearly visible. The big advantage of fluorescent tracers is however that fluorophores of different excitation and emission wavelengths can be combined in the same tissue, thus allowing to distinguish multiple populations of neurons by imaging with the appropriate wavelengths. Fig. 2.2 is a maximum intensity projection of a confocal stack of HVC acquired in an animal which was injected with 3 different tracers. Images of the different channels were acquired sequentially, to avoid simultaneous detection of fluorophores with similar spectra. In RA we injected Lucifer Yellow, in Area X we injected Alexa Fluor 647, and in Uva we injected Fluoro-Ruby. In each case, around 0.5 µl of tracer was used. The injections resulted in discrete labeling of the different populations so that
they were clearly distinguishable. Different quality of staining for the different tracers were observed, with staining of axons and dendrites in the case of Alexa Fluor 647 injected in Area X and Fluoro-Ruby injected in Uva, and staining mainly in the neuron somata only for Lucifer Yellow injected in RA. We found HVCx projecting cell somata to be bigger than HVCRA somata and the two populations to be non-overlapping, similar to what has been reported in the literature (Wild et al., 2005).

The dataset was acquired with a high-aperture lens, a 63x, NA 1.3 glycerol objective, which for shorter wavelengths can achieve theoretical resolution values of around 200 nm lateral and of around 400 nm axial, values which are insufficient to resolve individual synapses.

Figure 2.1: Example of retrograde tracing in the LMAN nucleus. Fluoro-Ruby dextran was injected into RA and the animal was sacrificed after 5 days. Vibratome sections were stained with DAPI and imaged in a confocal microscope. A maximum projection of a confocal stack is shown. Red: RA-projecting LMAN neurons; blue: DAPI-stained cell nuclei. Scale bar: 70 µm.
Figure 2.2: Maximum intensity projection of a confocal image stack of part of HVC. Three different tracers were injected in afferent and efferent brain regions of HVC, revealing distinct neuron types. RA-projecting neurons filled with Lucifer Yellow are visible in red, Area X-projecting neurons filled with Alexa Fluor 647 in blue, and axons from Uva filled with Fluoro-Ruby in green. Scale bar: 50 µm.

2.2.2 Tissue embedding and sectioning

Due to the drawback of LM of not being able to reliably detect individual synapses, we complement LM with EM. We therefore process the tissue for electron microscopy. Different preparation approaches exist. Fixation of the living tissue can be done either chemically, by freezing the tissue, or by a combination of both. For our preparation protocol, we decided to use chemical fixation, dehydration and resin embedding of the tissue, because it results in tissue with good membrane staining that can be cut in serial ultrathin sections, and it allows for embedding of entire vibratome brain sections. We perfuse the
animal few days after the surgery (typically around 5) with a fixative solution composed of 2% paraformaldehyde and 0.075% glutaraldehyde. The perfusion is performed by first applying pressure for 1-2 min with a manual pump, and then by placing the fixative container 1-1.5 meters higher than the animal (experiments conducted by perfusing animals with different pressures have shown that best quality can be achieved with pressure of around 0.6 bar, data not shown). After perfusion, we cut the brain with a vibratome in 80 µm sections. These sections can be imaged in the LM, wide-field or confocal (resulting in images such as Fig. 2.1 and Fig. 2.2). Sections acquired with a wide-field LM are important to later relocalize the region of interest. Preparation of the tissue for electron microscopy, as it will be discussed later, leads to strong bleaching of the tracer fluorescence, preventing localization in an embedded vibratome section of a brain region which was previously fluorescent. For this reason we acquire images of both transmitted light, which clearly shows blood vessels as well as other morphological landmarks which will still be visible after embedding, and fluorescent light, which shows tracer labeling. Overlay of the two image types allows to localize the fluorescent region relative to blood vessels and other landmarks, so that transmitted light images can be later used to resect the correct region when preparing for ultrathin sectioning (Fig. 2.3).

In addition to these reference images, confocal images can also be acquired at this point (e.g. Fig. 2.2). Since many different fluorophores can be present in the tissue, images are best acquired sequentially, 1-2 channels during each scan. This is necessary to optimize the excitation wavelengths and detection spectra, because with 4-5 fluorophores excitation spectra of some fluorophores often lie within the emission spectra of others. After LM imaging, the sections are prepared for electron microscopy. We use a postfixation and staining protocol based on reduced OsO₄ followed by normal OsO₄ and uranyl acetate, which has been shown to result in good fixation and high membrane contrast in brain tissue (Harris, Jensen, and Tsao, 1992; Knott et al., 2008). We then completely dehydrate the tissue with a graded series of ethanol followed by propylene oxide and we embed the brain in the epoxy resin Durcupan, which is then thermally cured. Fig. 2.4 shows an example of zebra finch tissue prepared with this protocol. The membranes are well stained and contrasted, and the ultrastructure is well preserved (notice e.g. the preservation of synaptic vesicles). After polymerization of the resin, we resect from the hardened sections the region to be further imaged. Transmitted light images previously acquired are used as a reference to correctly localize the region of interest and to trim the block to a size which can be cut in ultrathin sections (generally less than 1 mm², Fig. 2.3). We then cut the trimmed block in ultrathin sections using a diamond knife in an ultramicrotome. We collect the sections on different substrates depending on
Figure 2.3: Preparation of a block for ultrathin sectioning. A) Transmitted light image of HVC, marked with white arrows. Fluorescence imaging of the same section (not shown) confirmed HVC location. B) Transmitted light image of the same section after embedding. Due to embedding, contrast of the specimen changes, with loss of some reference points. Blood vessels, e.g. the one marked with a black arrow, are still visible and can be used to localize the region of interest. C) Transmitted light image of a block resected from the embedded section prior to trimming. D) Transmitted light image of the trimmed block. The blood vessel within HVC previously observed (black arrow) is still visible.
the electron microscope later used. Sections that are imaged with a TEM are collected on pioloform-coated grids, on which only few sections can be placed. For collection of longer arrays of sections and easier handling during successive immunostaining we use silicon wafers. Silicon wafers are semiconductor made of pure crystalline material. These properties give them the advantage of being a good substrate for SEM, because their electrical properties prevent charging of the probe and because their perfectly flat and regular surface prevent artifacts in particular when using a secondary electron detector, which is sensitive to the topology of the specimen. We use silicon wafers fragments with areas of up to 2-3 cm$^2$, on which arrays of several tens of sections can be placed.

![Transmission electron micrograph of zebra finch HVC prepared with reduced OsO$_4$, OsO$_4$ uranyl acetate, and embedded in Durcupan resin (see chapter 2.4 for preparation protocol). The preparation protocol with reduced OsO$_4$ results in well contrasted membranes. Scale bar: 1 μm.](image)

**Figure 2.4:** Transmission electron micrograph of zebra finch HVC prepared with reduced OsO$_4$, OsO$_4$ uranyl acetate, and embedded in Durcupan resin (see chapter 2.4 for preparation protocol). The preparation protocol with reduced OsO$_4$ results in well contrasted membranes. Scale bar: 1 μm.

### 2.2.3 Light microscopy of ultrathin sections

After collecting ultrathin sections, we image them with a wide-field light microscope. Although strongly reduced, we can still observe tracer fluorescence in the ultrathin sections for some tracers. This was the case for Texas-Red dextran and Alexa Fluor 488 dextran. In the case of HVC neurons retrogradely labeled with Alexa Fluor 488, the fluorescence was localized in clusters of small (less than one μm) fluorescence points with a size similar to a cell body (Fig. 2.5, top). Because of the weakness of the signal, with our setup (Olympus BX61, Olympus MT20 light source, F-View II camera) exposure times of at least one
second are required, making localization and focusing of the sections on the silicon wafer difficult. To facilitate this task, use of high-aperture oil lenses, DAPI staining of the ultrathin sections and initial localization of the sections with transmitted light can help. Moreover, the silicon wafer acts as a reflective surface. This optical property allows to collect more signal from the specimen, because part of the fluorescent light emitted in the direction opposite to the camera is reflected back. Detection of direct fluorescence in ultrathin sections collected on pioloform grids is more difficult. Due to the fragility of the sections, they can not be embedded in a mounting medium (this causes tension on the pioloform) and covered with a cover glass, which is necessary for high-aperture oil lenses. Moreover, even air lenses with high magnification and aperture have a very limited depth of field. If the pioloform sheet is not completely flat, focusing of the sections, which is already challenging due to low intensity of the signal, becomes very difficult, because in the same field of view some parts can be in focus whereas others are not. After imaging the direct fluorescence, sections can be further immunostained to detect bleached fluorophores. In the case of the probe shown in Fig. 2.5, which was placed on pioloform grids, we directly imaged it in the transmission electron microscope to identify the structures in which the fluorescent signal was localized (the only additional step between the two microscopy sessions was poststaining of the grids with lead citrate to further increase membrane contrast). Using section borders and blood vessels as a reference, we located the same region (Fig. 2.5, middle). Interestingly, superposition of LM and EM images (Fig. 2.5, bottom) reveals that the fluorescent point correspond to specific structures within a cell body. It is interesting to notice how this soma, belonging to an HVC_X neuron, is part of a cluster of neurons which are in tight contact (see also section 2.2.4). The large number of fluorescent points which corresponded to specific structures in the soma helped in the alignment of the two images, because they could also be used as reference points for fine adjustment of the alignment (which was done linearly). We speculated that these structures could be lysosomes. Whereas Alexa Fluor 488 fluorescence only survives embedding in lysosomes, fluorescence of Texas Red is more resistant and is still detectable in broader regions, corresponding to cell somata (Fig. 2.6, in this case HVC_{RA} neurons). Observation of images acquired with a confocal microscope after vibratome sectioning reveals that the tracer was previously localized in the whole cell body, as well as in proximal processes, but was already concentrated in compartments, in particular in the case of Alexa Fluor 488, for which small regions of high fluorescence signal are visible also along processes (Fig. 2.7, injection made by M. Kirschmann, the image is of the same brain as the section of Fig. 2.2).
Figure 2.5: Alexa Fluor 488 post-embedding fluorescence in HVC ultrathin sections of an animal which was injected with Alexa Fluor 488 in Area X. Top: Fluorescence light micrograph of a section containing fluorescence signal. Middle: transmission electron micrograph of the same section. Bottom: superposition of the light and electron micrographs. Fluorescence can be found within a soma, the nucleus of which is not visible in this section. Scale bar: 2.5 µm.
Figure 2.6: Texas Red direct fluorescence in HVC ultrathin sections of an animal which was injected with Texas Red in RA. In addition to the tracer-filled vesicles, similar to those observed with Alexa Fluor 488, the shape of the cell bodies is visible. Scale bar: 25 µm.

Figure 2.7: Confocal stack maximum projection of Texas Red and Alexa Fluor 488 dextran tracer fluorescence in HVC. Texas Red (red) dextran was injected in RA, Alexa Fluor 488 (green) in Area X. Both tracers are localized in compartments within the neurons, but also generally stain the whole cell body, especially Texas Red. Scale bar: 15 µm.
2.2.4 Clustering of projection neurons in HVC distinguished by correlative microscopy

Imaging of direct post-embedding fluorescence in ultrathin sections prior to EM imaging allows to gain information about the identity of cells in the tissue, without needing to image large volumes to have reference points necessary to match electron micrographs with pre-embedding confocal data (e.g. blood vessels). The following example shows this advantage in the case of HVC projection neurons. In electron micrographs of zebra finch brain, we frequently observe clusters of somata, typically consisting of 2-3 somata contacting each other in a single ultrathin section (Fig. 2.8, top; Fig. 2.5). Neighboring cells are in tight contact, with the plasma membrane of the cells separated only by extracellular space or even directly touching (Fig. 2.8, bottom). It should be however noted that chemical preparation of the tissue probably leads to changes in the volume of the extracellular space, and therefore it is difficult to assess the effective distance between neighboring cells.

Combination of light and electron microscopy of ultrathin sections allows us to classify neuron somata. Fig. 2.9 shows an example of two neighboring somata in direct contact. The animal was injected with Texas Red dextran in RA and Alexa Fluor 488 dextran in Area X, and after embedding we imaged direct fluorescence in HVC. Superposition of the light micrographs with the electron micrograph reveals that the two somata project to two different areas, RA and Area X. The two somata are in tight contact, with a significant part of the membranes directly touching (Fig. 2.9 bottom).

2.2.5 Immunostaining of ultrathin sections

Embedding of the probe with the chemical preparation previously described leads to reduction of the fluorescence in the tissue. For some tracers, this reduction results in complete loss of the signal, in particular if these only label processes and not somata in the area of interest. Tracers should however still be present in the tissue, considering that they are fixed in situ because of lysine residues incorporated into the dextran conjugate (lysine residues are fixed by aldehydes), that they are still visible after perfusion and that some fluorescence is still detected at the right position after embedding (Fig. 2.5). We therefore apply an immunohistochemical approach on ultrathin sections to localize tracers with quenched fluorescence. Unlike most immunohistochemical studies involving electron microscopy, but similar to (Micheva and Smith, 2007), we use fluorescent secondary antibodies instead of gold antibodies. The use of a fluorescence staining has for our purposes a number of advantages, concerning the number of different antibodies that can be used, the speed of imaging, and the
Figure 2.8: Top: Low magnification electron micrograph of HVC. Clusters of neuron somata can be often observed (white arrows). Scale bar: 10 µm. Bottom: Higher resolution micrograph of two neurons (N1 and N2) directly contacting each others. Note the electron-dense structures inside neuron N2 (black arrow), probably due to high concentration of tracer. Scale bar: 2 µm
Figure 2.9: Top: Light micrograph of neurons in HVC composed of two channels, green corresponding to a tracer injected in RA, red to a tracer injected in Area X. Direct post-embedding fluorescence was imaged, without immunostaining. Bottom: TEM micrograph of the corresponding region with fluorescence information overlaid. The cell somata in direct contact belong to distinct populations, one containing green staining and thus projecting to RA (white arrow) and the other containing red staining and projecting to Area X (black arrow). Scale bar: 5 µm
processing and correlation of the data acquired with LM and EM (see section 2.3).

Before incubating the sections with antibodies, we treat the sections with different chemicals (Brorson, 1998; Shi, Cote, and Taylor, 1997). In the case of TEM grids, we use periodic acid, whereas in the case of SEM sections (the type of section which we are currently using), we use sodium metaperiodate (periodic acid excessively decreases the contrast). After treatment of the sections with periodic acid or sodium metaperiodate, we incubate them with a blocking solution followed by incubation with highly-concentrated (1:50) primary antibodies. Because silicon wafers have a hydrophobic surface, drops of water solutions placed on them stay in place and therefore only small amounts of liquid are necessary, making incubation of arrays possible without needing excessive quantities of antibodies. Primary antibodies are washed and sections are incubated with fluorescent secondary antibodies, which are finally washed off. TEM grids are imaged with air lenses because embedding in a mounting medium risks to distort or damage the pioloform sheet (see chapter 2.2.3), whereas sections on silicon wafers can be embedded in a mounting medium that keeps the sections wet, covered with a cover glass and imaged with high-aperture oil lenses. We automate acquisition of images using the software package "Experiment Manager" of the CellR software on our Olympus BX61 microscope (the same procedure can be applied for acquisition of direct fluorescence described in section 2.2.3). The microscope has a high-precision motorized stage which can be programmed to acquire arrays (e.g. 3 x 3) of images covering parts of or complete ultrathin sections, for multiple consecutive sections whose position can be defined prior to acquisition of the dataset. Additionally, the microscope can be programmed to refocus before acquiring each set of images (or even each individual image), although autofocus correctness strongly depends on the contrast of the sections and on the presence of artifacts, unbound antibodies or dust over the sections (autofocus on our system cannot be used for direct fluorescence, because of very low light intensity). Care has to be taken to calibrate the stage so that it is as parallel as possible to the camera, because a deviation of only a few degrees already cause part of an image acquired with a 60x, NA 1.43 lens to be out of focus. We perform this operation manually, but it would be of great utility to develop methods to automate it.

Fig. 2.10 shows the result of immunostaining against a tracer and compares it to preembedding images of the same tissue. After preparing the tissue for electron microscopy and placing ultrathin sections onto TEM grids, we performed immunostaining against Lucifer Yellow, which was injected in RA and therefore retrogradely stained cell bodies in HVC (the tissue is the same as shown in Fig. 2.2). We acquired images of the ultrathin sections with a 40x, NA 0.6 air lens.
The signal could be localized in somata-shaped structures and, within them, in small regions of high intensity, probably lysosomes. Additionally, signal could be observed in processes close to the somata and scattered in smaller points over the entire section. For comparison, we localized the same region in the confocal stack that we acquired before embedding the tissue. For this purpose, it was important to keep track of the position of the area that was imaged prior to embedding (which was smaller than HVC) within the block. In this case, a blood vessel located in the center of the upper border of Fig. 2.10C was particularly useful, because it was also visible in the ultrathin sections (in a different channel than the one associated with immunostaining). Fig. 2.10B depicts a single optical section of the confocal stack located approximately at the same position as the ultrathin section (it should be noted that the optical section encompasses a larger volume than the volume associated with the ultrathin sections, due to low axial resolution of light microscopy). Localization in the z-plane was done such that the two cell bodies in the center of the immunostained section are present in the optical section together with their proximal processes. Because the optical section may not be parallel to the ultrathin sections (the optical section was parallel to the imaging plane and no resampling was done to correct for mismatch in parallelism between the confocal and the immunostaining imagery), not all the labeled structures are corresponding in the two images. Moreover, the tissue undergoes morphological transformations during preparation, mainly due to additional fixation, dehydration and embedding of the tissue, which further decrease the correspondence of the two images. Despite the rough alignment procedure, the comparison gives an impression of the quality of immunostaining, which allows to clearly identify the shape of the HVC

RA neuron somata as well as parts of their processes.

After all LM micrographs are acquired, including possible restaining after elution (section 2.2.11), we remove the cover glass from the wafer, clean the sections with distilled H$_2$O and dry them.

2.2.6 Electron microscopy imaging

Sections to be imaged in the EM are poststained with different chemicals, UAe, OsO$_4$ and lead citrate in different combination depending on the EM (TEM or SEM). These chemicals have the effect of increasing contrast, compensating for the loss of it which occurs due to resin etching, OsO$_4$ removal and immunostaining. The ultrathin sections are then imaged in the EM. Similar to EM acquisition for sections which contained direct fluorescence (see section 2.2.3), we localize the region of interest in the EM. This is done by first looking at low magnification at the position and orientation of the ultrathin sections, and then
Figure 2.10: Comparison of post-embedding tracer immunolabeling and pre-embedding confocal microscopy. (A) Immunolabeling of Lucifer Yellow tracer on ultrathin HVC section prepared on TEM grid. (B) Confocal optical slice of the same region as in (A), imaged after vibratome sectioning and prior to preparation of the tissue for TEM imaging. The selection of the optical section was based on the shape and location of the cells in the white dashed rectangle (no corrective image transformations were performed). (C) Maximum projection of a confocal image stack of 60 optical sections. Image adapted from (Oberti et al., 2010). Scale bar: 25 µm.
by increasing the magnification using section borders, folds and blood vessels in the tissue as a reference. In HVC, cell somata can also be used as reference, because their shape and relative position is easily recognizable in the EM as well as in the LM, if fluorescently labeled.

2.2.7 LM-EM correlation

We imaged the probe shown in Fig. 2.10 in the TEM at different magnifications, concentrating on the two somata-shaped fluorescent regions located in the middle of the probe (Fig. 2.11A). Electron microscopy of the section allowed us to identify at the ultrastructural level in the same region two cell somata, based on the presence of their cell nuclei, organelles in the cytoplasm and cell membranes delimiting the somata (Fig. 2.11B). The electron micrograph was aligned after imaging, starting with low magnification EM micrographs, based on features independent of the immunostaining signal (section borders, blood vessels, not visible in this cropped image) and also on features visible in the immunostaining signal. In particular, some immunostained, tracer-filled lysosomes are clearly visible in the electron micrographs as relatively big non-electron dense vesicles (or, in some cases, smaller and more electron dense). Thanks to their visibility in both images, these vesicles were also used to align the images. The probe, whose resin was etched with periodic acid prior to immunostaining and poststained before EM, has good membrane contrast, allowing to distinguish details already at low resolution, for example myelinated axons and other large processes. Fig. 2.11C shows the superposition of the light and electron micrographs. The fluorescent signal is nicely contained within the borders of the somata. Even small depressions in the cell membrane visible in the electron micrograph can be found in the immunostaining images (e.g. on the left side of the left cell). Thanks to the fluorescence image, the two cell bodies identified in the electron micrograph can be classified as HVC_RA projection neurons. One HVC_RA soma has a round section and does not have neighboring cells in direct contact, the other soma has a more elongated section and has direct contact with another unlabeled neuron. In the neuropil between the two soma thickly myelinated axons can be seen.

After acquiring the low magnification EM images in Fig. 2.11, we concentrated on features smaller than cell bodies to test if immunostaining signal could also be associated with processes and synapses imaged in the EM. We first imaged part of Fig. 2.11 at higher resolution to see if the fluorescence signal located on the right of the left HVC_RA neuron corresponded to an identifiable structure in the EM (Fig. 2.12). In the LM, two elongated regions of contiguous signal of varying intensity could be detected (Fig. 2.12A, asterisk and cross).
Figure 2.11: Correlation of LM and EM images of the same probe. (A) Light micrograph of the two cell bodies immunostained for Lucifer yellow shown in Fig. 2.10. (B) The same region as in (A) is imaged in the TEM, revealing two cell somata at the positions expected from the immunostaining. (C) Immunofluorescence image superimposed on the TEM image. The fluorescence signal matches with the shape of the cell somata visible in the electron micrograph. Image adapted from (Oberti et al., 2010). Scale bar: 5 µm.
Observation of the same position in the EM (Fig. 2.12B) and overlay of the two images (Fig. 2.12C) reveals that both fluorescent regions correspond to two continuous structures (Fig. 2.12C, asterisk and cross). The modulation in signal intensity seems to depend on the presence of intracellular organelles, in particular mitochondria, and the shape of the fluorescent region matches the shape of the processes.

We further increased magnification to better visualize synapses. We expect signal within synapses to be spatially highly restricted, and therefore more difficult to distinguish from unspecific staining, and present in consecutive sections due to ultrathin sectioning of the tissue (synapses is larger than the section thickness). We acquired LM and EM images of pairs of consecutive sections. As expected, some synapses were labeled in consecutive sections. In Fig. 2.13 two synapses are shown (A and B), each in two consecutive sections (1 and 2). The fluorescence signal corresponded well in both cases with a postsynaptic neuron (similar as before, the alignment was based on section borders, blood vessels and cell bodies). Ultrastructure preservation and contrast allowed us to detect membranes, synaptic vesicles, and allowed us to unambiguously identify synapses. One synapse on an HVC$\text{RA}$ neuron was part of a multi-synapse bouton that engulfed several spine heads, whereas the other synapse on HVC$\text{RA}$ neuron was made by a large bouton containing multiple mitochondria on an also relatively large process (Fig. 2.13).

2.2.8 Multicolor LM-EM correlation with immunolabeling

The greatest advantage of correlating light and electron imagery is that with light microscopy, unlike electron microscopy, it is possible to combine multiple markers to label in our case different neuron populations, as we have seen in section 2.2.4. In this section we show an example in which we combine direct post-embedding fluorescence with immunostaining on ultrathin sections to image with light microscopy two different cell populations and acquire electron micrographs of them. The animal was injected with several tracers. We concentrated on direct fluorescence of Texas Red dextran, which was injected in RA, and immunostained Lucifer Yellow dextran, which was injected in Uva (the other tracers were Fast Blue and Alexa Fluor 647 dextran, against which to our knowledge there is no antibody available, and Alexa Fluor 488, for which we also found an antibody which can detect it). The probe was embedded as previously described in the chapter and cut on silicon wafer. We imaged direct fluorescence of the ultrathin sections in the LM, acquiring images of Texas Red, whose fluorescence survived preparation and was visible in lysosomes as well as
Figure 2.12: Magnified view of part of Figure 2.11. (A) LM image of a dendrite immunostained for Lucifer yellow. (B) TEM micrograph of the same region as in (A). (C) Superposition of immunofluorescence and TEM images allows classification of the dendrite (asterisk) as belonging to an RA-projecting neuron. The tissue has good ultrastructure preservation and membrane contrast. Image adapted from (Oberti et al., 2010). Scale bar: 1 µm.
Figure 2.13: Correlative images of two synapses made by immunolabeled neurons. Both synapses (A and B) were imaged in two serial ultrathin sections (A1 and A2, B1 and B2, respectively). Top row: light micrographs of anti-Lucifer Yellow immunostaining. Middle row: transmission electron micrographs of the corresponding regions. Bottom row: superposition of light and electron micrographs. Fluorescence signal overlaps with a postsynaptic compartment and is present in both consecutive sections (A1, A2 and B1, B2 top). (A) The labeled spine is completely embraced by the presynaptic bouton, which also makes a synaptic contact with a second spine (asterisk in A1 and A2 bottom). (B) Many mitochondria are visible in the large presynaptic terminal (crosses in B1 and B2 bottom). Tissue preservation and contrast is sufficient to resolve synaptic vesicles (A and B middle and bottom). Image adapted from (Oberti et al., 2010). Scale bars: 250 nm.
in somata and in some processes. Thereafter we immunostained the probe to detect Lucifer Yellow, etching the probe with sodium metaperiodate and blocking unspecific binding with acetylated BSA. Although direct post-embedding fluorescence was still visible after immunostaining, we acquired it in a separate imaging session prior to immunolabeling. The reason is that immunolabeling is much stronger that direct fluorescence (few tens of milliseconds versus 1 second or more of exposure time), and therefore immunolabeling signal tends to be visible while imaging direct fluorescence even if different filters are used. The only drawbacks of this procedure, which we normally follow for all probes, are an increase in the time needed to image the probe and to align the images, because the same position has to be located twice (an operation which requires several seconds for each section) and images of the same position have to be aligned, e.g. in TrakEM2.

After acquiring LM and EM micrographs of the same region, we aligned the two fluorescence channels with the EM data (Fig. 2.14). The color information allowed us to classify distinct neural parts belonging to the two different neuron populations, HVC<sub>RA</sub> and Uva<sub>HVC</sub>, within the electron micrograph, including synapses (Fig. 2.15A and B) and somata (Fig. 2.15C and D). HVC<sub>RA</sub> signal was present in a soma and a relatively large unmylelinated processes, whereas Uva<sub>HVC</sub> signal was visible in presynaptic terminals with thick synaptic membrane. Similar as before, we compared pairs of consecutive sections to test if signal at the synapses and other processes was present in both sections, which we found to be the case (Fig. 2.15A and B, C and D). Moreover closer inspection of the tissue reveals that this preparation done on silicon wafer and using SEM instead of TEM also results in good ultrastructure quality and membrane contrast, allowing to distinguish small processes (Fig. 2.16).

2.2.9 HVC<sub>RA</sub> projection neurons

The preparation described in the previous sections allows us to trace in the same tissue volume multiple populations of neurons, for example by classifying two populations by direct fluorescence in ultrathin sections (as in Fig. 2.9) and an additional one by immunolabeling. In the following examples, we took advantage of good preservation of Texas Red dextran fluorescence to better characterize HVC<sub>RA</sub> projection neurons, and of immunolabeling of the same tissue to describe Uva<sub>HVC</sub> synapses in HVC.

We reconstructed part of the soma and the proximal part of a dendrite of an HVC<sub>RA</sub> neuron based on ultrathin serial sections that we imaged both in the LM and in the EM. LM information allowed us to identify the soma and proximal processes of the neuron (Fig. 2.17A-C) and select the regions to image in the
Figure 2.14: Correlation of multicolor LM and SEM images. Top: superposition of two fluorescence channels, associated with Texas Red dextran in HVC_{RA} cells (red) and anti-Lucifer Yellow immunostaining in Uva_{HVC} neurons (yellow). Bottom: superposition of light micrographs with low resolution scanning electron micrograph of the same region. Fig. 2.15 and 2.16 show higher-magnification images of the same tissue. Scale bar: 8 µm.
EM. After EM imaging, we aligned light and electron micrographs and traced the identified HVC<sub>RA</sub> neuron in serial sections with TrakEM2 software (Fig. 2.17D-G). For better reconstruction results, we aligned consecutive SEM micrographs with an elastic registration algorithm in TrakEM2 (elastic spring-mesh registration) which registers the image with a range of images before and after every section. The proximal dendrite appeared to be predominantly smooth, similar to observations in the canary (Canady et al., 1988). We then reconstructed two synaptic contacts on the dendrites and part of the corresponding boutons (Fig. 2.17G, blue and green).

The first synaptic contact we reconstructed, closer to the soma, was part of a large bouton (more than 1 µm in diameter, part of the bouton was outside of the imaged volume, Fig. 2.18). This bouton contained at least four synaptic contacts at different positions on its surface. These synapses were all similar in appearance, with vesicles of irregular shape and pre- and post-synaptic thickenings of similar dimensions (Fig. 2.18B), characteristics of Gray’s type II (or symmetric) synapses (Gray, 1959). Although immunolabeling would be necessary to confirm the chemical nature of these synapses, based on observation in other animal models it is reasonable to speculate that they are inhibitory (Kleemann and Roubos, 2011).

We reconstructed a second synaptic contact more distal from the soma (Fig. 2.19). The reconstructed presynaptic neuron was also large in size and made two synaptic contacts with other partners in close proximity to the synapse with the HVC<sub>RA</sub> dendrite. These contacts were also symmetric, with non-prominent postsynaptic density. Again, it would be interesting to reconstruct further parts of the presynaptic neuron, test whether the synapse is inhibitory and belongs to an interneuron, and identify the postsynaptic partners.

**Figure 2.15 (facing page):** Correlation of EM and multicolor LM micrographs of consecutive ultrathin sections. Left column: light micrographs of two different projection neuron populations, HVC<sub>RA</sub> neurons labeled in red with Texas Red-dextran (direct post-embedding fluorescence) and Uva<sub>HVC</sub> axons in yellow with anti-Lucifer Yellow immunostaining. Right column: immunofluorescence micrograph superimposed on SEM micrograph of the corresponding position, allowing for classification of synaptic terminals (black arrows in A and B), small neural processes (white arrows in C and D, asterisk in A and B), and cell somata (asterisks in C and D). A and B, as well as C and D are images taken from adjacent section pairs: immunofluorescence signal on neighboring sections coincides with the same structures as identified in the EM. The region enclosed by the dashed square is shown in higher magnification in Fig. 2.16. Image adapted from (Oberti et al., 2011). Scale bar: 1 µm.
Figure 2.16: Magnified micrograph of the region in Fig. 2.15 enclosed by the dashed square. The good preservation of ultrastructure and contrast is illustrated by membranes of small neurites that have no gap artifacts and appear fully closed (white arrows) and by highly contrasted synaptic densities (black arrows). The fluorescence signal seems to extend outside the border of the neurons, due to the strong signal and low resolution of LM (around 200 nm at best). Image adapted from (Oberti et al., 2011). Scale bar: 500 nm.
2.2.10 Uva\textsubscript{HVC} neurons

HVC receives thalamic input from the nucleus Uva, which is its only thalamic afferent (see chapter 1.1). Using our correlative array tomography approach, we imaged and reconstructed synapses made by Uva\textsubscript{HVC} neurons in HVC. We were able to do so in the tissue used for the previous section, because the tissue also contained a tracer which had been injected in Uva. It should be noted that although histological examination of the sections after vibratome sectioning confirmed that the injection was localized in Uva (Fig. 2.20), we found few cells retrogradely labeled in HVC. No retrograde projection from HVC to Uva has been reported in the literature, and we think that the labeling may be due to labeling of axons belonging to other neuron populations along the pipette tract or to an unknown projection from a region close to Uva.

The first synapse we reconstructed was part of a relatively large bouton visible in around 10 consecutive ultrathin sections (Fig. 2.21A-F). The bouton only contained one synaptic contact with a postsynaptic partner (Fig. 2.21G, red) which also received in close proximity input from three other synapses (Fig. 2.21I). The vesicles in the Uva\textsubscript{HVC} neurons were round and a strong electron-dense staining of the synaptic membranes was visible (Fig. 2.21H). We classified the synapse as Gray’s type I (asymmetric). Interestingly we noticed a large intracellular compartment in the bouton, which was devoid of tracer (Fig. 2.21D and E). In another case, we also observed a large presynaptic bouton containing an asymmetric synapse on a large postsynaptic neuron (Fig. 2.22, in this case we did not reconstruct the bouton because it was at the border of the imaged volume).

The second synapse we reconstructed was also part of a bouton with a single synaptic contact (Fig. 2.23). We reconstructed both the pre- and the postsynaptic partners close to the synaptic contact, and noticed that they tended to

\textbf{Figure 2.17 (facing page):} Correlative imaging and reconstruction of an HVC\textsubscript{RA} neuron. (A-C) Light micrographs of ultrathin sections of an HVC\textsubscript{RA} neuron labeled after injection of Texas Red dextran in RA. The signal corresponds to direct fluorescence detected after embedding and prior to immunostaining against a second tracer present in the tissue. Signal can be detected both in vesicles with high concentration of tracer and in the soma and parts of dendrites (white arrow in (B)). The three sections were taken at different z-positions. (D-F) Electron micrographs of the regions imaged in the LM shown in (A-C). The labeled neuron (red), its nucleus (violet), as well as two synapses (blue and green) were traced with TrakEM2 software. The same dendrite marked in (B) is also visible in EM imagery (E, white arrow). (G) Three-dimensional reconstruction of the labeled HVC\textsubscript{RA} neuron, based on ultrathin serial sections manually segmented with TrakEM2 software. The processes were reconstructed until the asterisk symbol. The two synapses highlighted in blue and green are shown at higher magnification in Figs. 2.18 and 2.19. Scale bars: 5 µm.
Figure 2.18: Reconstruction of a synaptic contact on the proximal HVC_{RA} dendrite shown in Fig. 2.17. (A) Three-dimensional reconstruction of the HVC_{RA} dendrite (red) and synaptic bouton (blue). Synaptic contacts made by the bouton in the reconstructed volume are labeled in yellow. (B) High-magnification image of the synaptic contact made with the HVC_{RA} dendrite. The synapse appears to be of symmetric type, lacking a prominent postsynaptic density. (C-D) Electron micrographs of the bouton at different depths. The HVC_{RA} dendrite and the bouton were segmented and labeled (red and blue). The bouton, containing multiple mitochondria, is large in size and in addition to the synapse made with the identified dendrite (white arrows) also potentially contacts other neurons (green arrows). Scale bars: (B) 250 nm; (C-F) 500 nm.
Figure 2.19: Reconstruction of a second synaptic contact on the HVC\textsubscript{RA} dendrite shown in Fig. 2.17. (A) Three-dimensional reconstruction of the HVC\textsubscript{RA} dendrite (red) and synaptic bouton (green). Synaptic contacts made by the bouton in the reconstructed volume are labeled in yellow. (B) High-magnification image of the synaptic contact made with the HVC\textsubscript{RA} dendrite. This synapse also appears to be of symmetric type, lacking a prominent postsynaptic density and having vesicles of irregular shape. (C-H) Electron micrographs of the bouton at different depths. The HVC\textsubscript{RA} dendrite and the bouton were segmented and labeled (red and green). The bouton, similar to the one in Fig. 2.18, is large in size and makes synaptic contacts with other neurons (black arrows) in addition to the synapse with the identified dendrite (white arrows). Scale bars: (B) 100 nm; (C-H) 500 nm.
wrap around each other (Fig. 2.23G). We also noticed an intracellular compartment, similar to the first reconstructed bouton (in this case however due to a fold in a section we could not exclude that the compartment was part of a different neuron engulfed by the UvaHVC neuron). The synapse also appeared to be asymmetric, although the shape of the vesicles was not clearly distinguishable (Fig. 2.23H).

Whereas in the three previous cases the synaptic contact was part of a large presynaptic bouton, in another case we observed that the synapse was made along the axon, which did not have a larger diameter than away from the synapse (Fig. 2.24A). In contrast, the postsynaptic neuron tended to wrap around the UvaHVC axon (Fig. ), and also formed another synapse in close proximity with another neuron. The synaptic contact was smaller in size and had less vesicles than in the previous two cases, but was also asymmetric.

### 2.2.11 Antibody elution

We identified 3 combinations of tracer and antibody reliably working: Lucifer Yellow, Texas Red, and Alexa Fluor 488. Unfortunately, all three antibodies commercially available were raised in the same species, therefore hindering simultaneous immunolabeling. We applied antibody elution as proposed by Micheva and Smith, in order to remove antibodies to perform a second immunostaining on a previously immunolabeled section (Micheva et al., 2010). In the case presented here, we eluted anti-Lucifer Yellow antibodies to stain Alexa Fluor 488 (in addition we imaged Texas Red postembedding fluorescence prior to immunostaining).

We acquired three micrographs of each section, one before immunostaining of direct Texas Red postembedding fluorescence (Fig. 2.25, red), one after the first
Figure 2.21: Correlative imaging and reconstruction of a synapse made in HVC by Uva-projecting neuron. (A-F) Left column: fluorescence imaging of an immunostained Uva<sub>HVC</sub> axon. Right column: electron micrographs of the corresponding image superimposed on the fluorescence micrographs. Six sections at different depths are shown, with the synaptic contact marked with black arrows. The fluorescence signal nicely overlays the inside of the axon, with regions containing mitochondria or other organelles showing less fluorescence signal (e.g. central region in (B) and the region marked with an asterisk in (E)). (G) Three-dimensional reconstruction of the synaptic bouton (yellow) with the synaptic contact labeled in red. The axon was reconstructed until the point marked with the asterisk. (H) Detail of the synapse, showing a prominent postsynaptic density and rounded vesicles (black arrows), typical of an asymmetric synapse. (I) In addition to the synapse from the Uva<sub>HVC</sub> neurons (cross), other asymmetric synapses are visible on the same postsynaptic partner (white and black arrows). Scale bars: (A-F and I) 500 nm; (H) 100 nm.
immunostaining against Lucifer Yellow (Fig. 2.25, blue), and one after elution and the second immunostaining against Alexa Fluor 488 (Fig. 2.25, green). After acquiring all light micrographs, we washed the sections and poststained them for electron microscopy. We used a combination of 1 min 1% OsO₄, 30 min 5% uranyl acetate, and 3 min Reynold’s lead citrate, which resulted in a much stronger contrast of the sections (Fig. 2.25C) compared to unstained sections. We acquired images of the same regions and aligned them with the light micrographs (Figs. 2.25D and 2.25E). In this way, we were able to classify different neurons depending on their projection partner, e.g. somata of cells projecting to RA and Area X and axons from Uva (Fig. 2.25F), whose identity was confirmed by the presence of fluorescent signal on multiple sections. It should be noted that the ultrastructure contrast decreases due to the elution and multiple immunostaining procedure. With the poststaining we applied, we were able to recover contrast of membranes and synapses, although it was worse than tissue without elution and not sufficient for reliable segmentation. We observed an improvement in contrast thanks to poststaining, and we hope that it is possible to further improve contrast by optimizing the poststaining protocol or by changing immunostaining and elution protocol (e.g. by reducing elution time or changing blocking solutions, which may accumulate on the surface of the section).
**Figure 2.23:** Correlative imaging and reconstruction of a synapse made in HVC by an Uva-projecting neuron. (A-D) Electron micrograph of the synapse at different depths. The Uva$_{HVC}$ bouton (yellow, the dendrite was colored in TrakEM2), identified with fluorescence (E-F), and its postsynaptic partner (blue) were segmented and reconstructed. A prominent postsynaptic density is clearly visible (white arrows). (E-F) Light micrographs of the immunostained Uva$_{HVC}$ axons and corresponding electron micrographs (in this case the yellow color corresponds to the fluorescence signal). (G) Reconstruction of the synaptic bouton (yellow) and of its postsynaptic partner (blue). The two neurons tightly wrap around each others. (H) High-magnification view of the synaptic region. Staining at the synaptic contact is very strong (white arrows), however the shape of the vesicles (blue arrows) is not clearly distinguishable. Scale bars: (A-D) 500 nm; (H) 100 nm.
Figure 2.24: Correlative imaging and reconstruction of a synapse made in HVC by an Uva_{HVC} neuron. (A) Three-dimensional reconstruction of an Uva_{HVC} axon (yellow) making a synapse (red) with a dendrite (blue). The postsynaptic partner wraps around the axon. The neurons were reconstructed until the points marked with asterisks. (B-E) Left column: Light micrographs of immunostained Uva_{HVC} neurons. Right column: electron micrographs superimposed on the light micrographs (yellow) and with the postsynaptic partner additionally manually labeled (blue). The asymmetric synapse with the reconstructed postsynaptic partner is marked with white arrows, a second synapse made by a different neuron is marked with a black arrow. Sections (E) was taken approximately at the position marked with a white arrow in (A). The fluorescence signal was well visible along the axon. Scale bar: 500 nm.
Figure 2.25: Multiple immunostaining rounds with antibody elution. Green: anti-Alexa Fluor 488 immunostaining labeling HVCX neurons. Blue: anti-Lucifer Yellow immunostaining labeling UvaHVC axons. Red: Texas Red postembedding fluorescence corresponding to HVCRA neurons. (A and B) Overlay of the three light micrographs for two consecutive sections. (C) Electron micrograph of the tissue. (D and E) Overlay of the light micrographs (region enclosed in the white box in A) with the electron micrographs of the same positions. (F) Same section as in (E), highlighted are three identified neurons, two somata (green and red) and a process (blue). Scale bars A, B: 5 µm; C: 1 µm; D, E, F: 2 µm.
2.3 Discussion

In this section, we will first discuss specific aspects of the preparation protocol, for example the choice of chemicals used to fix and stain the tissue and alternative available methods (section 2.3.1). We will compare our preparation protocol and the resulting data with the original array tomography protocol proposed by Micheva and Smith, and address the importance of using fluorescent antibodies instead of following a classic immunogold protocol (section 2.3.2). We will then discuss how images acquired with LM and EM can be registered, what the reference points are and which solutions are available to automate identification of corresponding positions between the two microscopes (section 2.3.3). Next, we will compare our workflow with other established techniques, including other correlative approaches (section 2.3.4). Finally, we will discuss the data we obtained with our correlative array tomography regarding the songbird brain (section 2.3.5).

2.3.1 Preparation workflow

At the beginning of the project, we decided to combine light and electron microscopy to gain the advantage of both multicolor information, which allows us to distinguish and classify multiple population of neurons, and high resolution, to detect at the ultrastructure levels features such as synapses. We adapted correlative array tomography to our animal model, the zebra finch, by combining in vivo injection of tracers of different colors with light and electron microscopy of ultrathin serial sections. The main difficulty of combining fluorescence LM and EM is an incompatibility of tissue preparation. Fluorescence light microscopy requires light fixation of the tissue, to avoid bleaching or quenching of the fluorophores, whereas electron microscopy often requires strong fixation and staining to preserve and give contrast to ultrastructure. These conflicting requirements result either in good fluorescence signal but poor ultrastructure quality, or in good ultrastructure but decrease or loss of fluorescence.

We were able to overcome this difficulty by showing that the tracers previously injected in vivo can be detected in ultrathin sections either directly, or by immunolabeling them if their fluorescence is completely quenched by OsO₄ treatment. Preparation of the tissue with reduced OsO₄ and uranyl acetate, and embedding in epoxy resin preserved well the ultrastructure and membrane contrast, allowing for unambiguous identification of synapses. Improvement of ultrastructure quality thanks to reduced osmium, first introduced as a mixture of potassium-ferrocyanide with OsO₄ by (Karnovsky, 1971), has been reported several times in the literature. The effects of potassium-ferrocyanide and potassium-ferricyanide on spinal cord tissue were investigated by (Langford and
Coggeshall, 1980), who concentrated in particular on preservation of myelin. They observed best quality with a mixture of OsO$_4$-potassium ferricyanide, and worse (but still better than with OsO$_4$ alone) results with OsO$_4$-potassium ferrocyanide. In this second case, membranes were also well preserved, but they found that the cytoplasm and nuclei were not fixed optimally. Rivlin and Raymond investigated the effect of different fixation mixtures on membrane fixation and contrast of retinal tissue that needed to be reconstructed from serial sections (Rivlin and Raymond, 1987). They postfixed the tissue in either OsO$_4$-potassium ferrocyanide, OsO$_4$-potassium ferrocyanide, or glutaraldehyde-tannic acid. They reported enhanced staining of the plasma membrane compared to OsO$_4$ alone with comparable results with all combinations of OsO$_4$ with potassium ferrocyanide/ferrocyanide tested, unlike the previously cited study by Langford and Coggeshall, which reported extraction of nuclei and less optimal preservation of the cytoplasm with potassium ferrocyanide compared to potassium ferricyanide (Langford and Coggeshall, 1980). The chemical mechanism for staining of biological tissue by OsO$_4$-potassium ferrocyanide mixtures has been investigated by (White, Mazurkiewicz, and Barnett, 1979). The authors found that potassium ferrocyanide provides reduction of OsO$_4$ leading to an equilibrium state in which Os is present at lower oxidation states which have higher reactivity than in OsO$_4$ and which tend to be chelated by atoms in the tissue. As a results, the presence of these Os forms leads to more Os deposition than OsO$_4$ alone.

In addition to increased membrane contrast, potassium ferrocyanide-reduced osmium has been shown to better retain cytochemical reactivity (Tamaki and Yamashina, 1994). The authors analyzed immunoreactivity of different antibodies in non-neural tissue (parotid gland, intestine, pancreas) embedded in LR White resin, finding that reactivity was better in tissue that was prepared with reduced OsO$_4$ compared to tissue post-fixed with conventional OsO$_4$ solution. The authors also noted that treatment of the ultrathin sections with sodium metaperiodate, an oxidizing agent, further increased labeling density, probably due to resin etching and bleaching of excessive OsO$_4$.

In addition to chemical fixation of the tissue, there are also physical methods to fix the tissue, for example by freezing it. An example of freezing is high-pressure freezing (Studer, Humbel, and Chiquet, 2008). In this technique, the specimen to be fixed is placed in a chamber that is first pressurized to over 200 MPa and within 20 ms cooled down to -196 °C (in some machines the same is achieved by shooting highly pressurized liquid nitrogen onto the probe). Freezing the tissue at high pressure prevents artifacts due to ice crystal formation, because high pressure leads to formation of vitreous ice. After cryofixation, the sample can be processed in different ways, such as freeze fracturing, cryosectioning, and freeze substitution. Freeze fracturing is often used to investigate membranes.
and distribution of transmembrane protein molecules. Cryopreparation is a purely physical preparation method, as the tissue is ultrathinly sectioned and imaged while frozen, and tissue prepared in this way is in principle imaged in a form which is most similar to the natural state, because no chemical modification is introduced by fixatives, stains, solvents, and resins. Whereas these two methods are not suited for investigating neural connectivity in large volumes of brain tissue, freeze substitution can result in tissue with high ultrastructural and staining quality (see (Frotscher et al., 2007) for excellent examples of micrographs of hippocampal slice cultures). In freeze substitution, high-pressure freezing is combined with resin embedding. The tissue is dehydrated at around -90 °C, and then treated with osmium tetroxide and embedded in a resin at increasing temperatures. Unfortunately, this approach suffers from a major limitation in the size of the sample that can be high-pressure frozen. These can have maximal thickness of 200 µm and a diameter of 1.3-3 mm (Studer, Humbel, and Chiquet, 2008), thus requiring very fast and precise dissection of the area of interest in the unfixed brain, an almost impossible task.

The other approach to tissue preparation, which we used in the present work, is chemical fixation. In chemical fixation, the organ to be prepared is perfused with a chemical fixative such as paraformaldehyde or glutaraldehyde. After perfusion, the tissue can be processed in different ways. One possibility is to further cryofix the tissue and then embed it in a resin, thus continuing with the preparation protocol described above (e.g. cryofixation followed by freeze substitution, (Mühlfeld, 2010)). Alternatively, chemically fixed sections can be cryoprotected and frozen for cryosectioning, according to the Tokuyasu preparation (Tokuyasu, 1973), a preparation that is however not suited for serial sectioning due to the extreme fragility of ultrathin cryosections (see section 3.3 for this approach on zebra finch brain). Lastly, the method most frequently used for preparation of brains for connectivity studies is chemical fixation followed by dehydration and resin embedding at temperature above 0 °C (Harris, Jensen, and Tsao, 1992; da Costa and Martin, 2011; Briggman, Helmstaedter, and Denk, 2011; Knott et al., 2002; Knott et al., 2006; Bock et al., 2011). With this method the perfused tissue is typically cut in sections with a thickness of around 80 µm, postfixed and stained with OsO₄ and uranyl acetate, dehydrated and embedded in a resin, e.g. the acrylic resin LR White or the epoxy resin Epon. Ultrathin sections with a thickness below 100 nm can then be cut with a diamond knife and imaged in a conventional TEM or SEM. With our preparation, we follow this type of chemical preparation. In particular, we use for initial fixation a mixture of paraformaldehyde and glutaraldehyde. Paraformaldehyde is a fixative which reacts primarily with -NH₂ groups and also with C=C groups, cross-linking proteins and fatty acids.
Paraformaldehyde has the advantage of fast penetration in the tissue, and has limited influence on antigenicity. Glutaraldehyde also reacts primarily with -NH₂ groups, penetrates slower in the tissue, but has stronger fixation effect and stronger detrimental effect on antigenicity (Hayat, 1989). Concentrations of as low as 0.5% glutaraldehyde can already cause a loss of 50% of the antigenicity of some molecules (Krähenbühl and Jamieson, 1973), however tissue fixed with glutaraldehyde shows superior morphological preservation than fixed with paraformaldehyde alone (Sabatini, 1963). For this reason, we use a mixture of the two fixatives (similar to (Karnovsky, 1965)), with a low concentration of glutaraldehyde, because we later perform postembedding immunostaining.

We identified 3 combinations of tracer and antibody that could be reliably detected. In addition, two tracers could be directly detected in ultrathin sections thanks to survival of their fluorescence. We tested an elution protocol in order to be able to use different antibodies on the same sections, but noticed a decrease in the ultrastructure contrast, which could only be partially compensated by poststaining of the sections. In order to be able to use several antibodies without having to elute them, it would be of advantage to produce anti-dye antibodies in different animals species. Alternatively, it would be be possible to combine fluorescent markers with an electron dense label, although in this case the embedding protocol may need to be changed (e.g. by omitting reduced OsO₄), with consequences on the ultrastructure quality.

### 2.3.2 Comparison with array tomography

Our preparation protocol, based on array tomography, differs in many points from the original array tomography protocol proposed by Micheva and Smith (Micheva and Smith, 2007; Micheva et al., 2010). In array tomography, the tissue is most of the times prepared without OsO₄ and only partially dehydrated (up to 95% ethanol), which allows to preserve fluorescence in the tissue. Moreover, for the same reason and for its advertised advantage for immunostaining, due to its hydrophilicity, the authors use the acrylic resin LR White. When doing correlative imaging, the authors also added in the preparation stronger fixation with glutaraldehyde and reduced OsO₄. With this preparation, as we mentioned in the introduction, the resulting ultrastructure quality is not sufficient for a reliable detection of synapses nor for tracing of neurons, which may be necessary if the fluorescent label is not distributed in the entirety of a neuron process (Fig. 1.4).

Several changes in our preparation protocol lead to the difference in ultrastructure quality. First, in our case, tissue is prepared with long incubations in reduced OsO₄, normal OsO₄, and uranyl acetate, whereas in array tomography
the tissue is only incubated in reduced OsO₄, for few minutes with microwave irradiation. Second, we dehydrate the tissue completely, whereas array tomography stops at 95% ethanol. Third, we use the epoxy resin Durcupan, in contrast to the acrylic resin LR White. In our experience, LR White tends to extract and damage the tissue more, whereas Durcupan results in better ultrastructure quality and is, in our case at least, compatible with immunolabeling. Third, we place the sections to be imaged in the SEM on silicon wafer, which, compared to coated glass, we found to give better results both in the LM, with more signal thanks to reflection of emitted fluorescence, and in the EM, with no topographic artifacts thanks to the perfect flatness of the substrate.

It should be noted that the difference in preparation and resulting tissue quality reflects the different needs of array tomography and of our correlative approach. Array tomography focuses on light microscopy and on the detection of multiple antigens, and is advertised as a proteomic imaging method. In their most recent publication, the authors applied array tomography to analyze synapses and their distribution in the mouse cortex, using various presynaptic, postsynaptic, and neurotransmitter-specific markers, in multiple rounds of staining with up to 17 markers for one area of interest (Micheva et al., 2010). In this sense, array tomography is a promising technique to reveal the molecular complexity of neurons. In our case, in contrast, good ultrastructure quality is essential, because we aim at unambiguous synapse identification and tracing of neurons with EM imagery. Although resolution values in array tomography are very high and could eventually suffice to distinguish synapses (which can have size of only few tens of nanometers), for connectivity studies they would need to be stained reliably with markers visible in the LM. To our knowledge, no staining technique has been so far able to label all synapses in a tissue without false positives nor negatives. Immunostaining suffers from the problem that antibodies can not penetrate completely thick tissue sections, resulting in different levels of labeling at different depths of the specimen, and that unspecific binding of antibodies results in false positive staining. Alternatively, viral tools could be used, for example vectors expressing a fluorescent marker tagging a synaptic protein such as synaptophysin (Roberts et al., 2008; Grinevich, Brecht, and Osten, 2005). Although this is very promising approach, it is not clear if all synapses of a virally infected neuron would be labeled, all neurons in the imaged volume would need to be infected, and it should be tested whether expression of the tagged protein reflects physiological levels and distribution.

The use of fluorescent antibodies, instead of gold antibodies as often applied in post-embedding immunohistochemistry, has a number of advantages. First, fluorescent antibodies are compatible with antibody elution (see section 2.2.11).
This procedure is not possible with gold-labeled antibodies for two reasons. First, this protocol requires the antibodies to stay in a water environment for the elution to work, whereas gold-labeled antibodies need to be imaged in the EM, and the probe has therefore to be dried after immunolabeling (thus hindering successive elutions). Second, if gold-labeled antibodies are used, their detection requires high-resolution imaging in the electron microscope. Irradiation of the probe with the electron beam leads to changes in the probe, thus affecting antigenicity of the tissue.

The second advantage of fluorescent antibodies is the speed of imaging. Gold particles conjugated to antibodies have different sizes, from few to many tens of nanometers, with antibodies conjugates to smaller particles resulting in higher labeling densities (Giberson and Demaree, 1994). As a consequence of the small size of gold conjugates, high-resolution EM imaging is needed, in particular if gold particles of different sizes need to be distinguished, with the consequence of longer imaging time in the EM. Fluorescent antibodies in contrast are imaged in the LM, which is significantly faster (less than 1 second for areas bigger than 100 x 100 \( \mu \)m\(^2\), and allows us to rapidly image many sections to select regions of interest which can then be imaged in the EM. Light microscopy has of course the disadvantage of lower resolution, 200 nm of lateral and 400 nm of axial resolution at best. Imaging of ultrathin sections however overcomes the limitation in axial resolution, bringing it to the same value of serial sectioning EM (FIBSEM and SBEM can reach better axial resolution by milling respectively cutting thinner layers of tissue than in classic ultrathin sectioning with an ultramicrotome). Regarding lateral resolution, EM allows to reach a resolution two orders of magnitude higher. The scope of the immunostaining we perform is however to determine if the tracer we used to label neurons is present in a specific neuron, and not to localize its exact intracellular position. Even if some parts of neuron processes can have a diameter smaller than 200 nm, these neurons can be traced in EM imagery over consecutive sections until a region with a diameter larger than the lateral LM resolution is reached, and this section of the neuron can be classified it depending on the presence of immunostaining signal.

The third advantage of fluorescently-labeled antibodies over gold-labeled antibodies is related to the correlation of LM and EM images and classification of the structures traced in the EM. If fluorescent antibodies are applied, classification of neurons depends on the presence of fluorescent signal inside them, which is easily detected by automatic methods (e.g. by binarizing the image with an appropriate threshold). Classification can be compared across consecutive sections. Gold particles conjugated to antibodies are in contrast more difficult to automatically detect, because their recognition must be based on their shape,
because they are represented in the same monochrome image as the biological tissue. Moreover, gold particles need to be counted, because background staining cannot be excluded (and therefore presence of gold particles at unspecific positions) and thus a decision about significant presence of signal has to be made based on the number of gold particles in the observed structure.

2.3.3 Image registration and localization of corresponding positions

During imaging, we manually located in the EM the same region previously imaged in the LM, based on several features such as sections borders, blood vessels, somata, and subcellular compartments. In particular, we found signal within structures which we identified as lysosomes. Tracer, pressure injected in the brain, is probably taken up and transported by neurons by active and passive processes. In the first case, the tracer presumably enters the endocytic pathway, thus ending up in lysosomes. Localization of tracer in lysosomes-like organelles has been previously reported after injection of Fluorogold. Fluorogold is a fluorescent dye introduced as a retrograde axonal tracer which provides retrograde filling of somata and proximal dendrites, does not label undamaged fibers of passage, and allows for long survival times (Schmued and Fallon, 1986). The authors noted that the retrogradely transported tracer, injected into different parts of the nervous system, is characterized by fluorescent granules within the cytoplasm and processes. Persson and Havton recently investigated the distribution of Fluorogold at the ultrastructural level (Persson and Havton, 2009). They examined localization of Fluorogold in spinal motoneurons after systemic delivery of the tracer by intraperitoneal injection. To this purpose, the sections were dehydrated and embedded in Lowicryl HM20 resin in the absence of OsO₄ using a freeze substitution protocol, and fluorogold was detected using immunogold labeling. EM imaging revealed that the tracer was localized principally in lysosomes. This result is similar to our observation, although in our case light microscopy of vibratome sections reveals that dextran fluorescent tracers tend to be distributed also in the whole cell body and not only in lysosomes (Fig. 2.2).

The process of finding corresponding positions in the LM and EM is slow and requires manual intervention each time a new position has to be found. Tools to semiautomatically find corresponding positions in the LM and EM are currently being developed by microscopy companies, Zeiss and FEI. The solution proposed by FEI, called Maps, is software based. Corresponding points within the probe which are visible in both LM and EM are associated in the software controlling the EM, in which it is possible to import LM images. By defining
two pairs of corresponding points, the coordinate system allows to directly move the EM to positions defined by clicking on the LM image. The approach proposed by Zeiss, called Shuttle&Find, comprises additionally a hardware part. The probe is placed on a special holder which contains three reference markers which are visible both in the LM and EM. These markers are used by the software controlling the two microscopes for calibration, so that it is possible to directly move the microscope to a specific position by clicking on an image acquired with the other microscope. This approach has the advantage that there is no need to find corresponding points within the probe, which would require imaging of the probe (with possible changes in the contrast of the tissue in the EM) and identification of features visible under both microscopes. Both methods have the advantages of drastically reducing the time needed to find the interesting region and of allowing automation of imaging. It is in fact conceivable to develop an algorithm which analyzes light micrographs and, depending on certain features (e.g. presence within a certain radius of fluorescence signal in two channels corresponding to different tracers), adds to a list the coordinates of regions to be imaged at synaptic resolution. This list can then be sent to the EM which, thanks to the calibrated coordinate system, can directly move to the correct section and position and image it, without human intervention except for moving the probe and loading it in the EM.

2.3.4 Comparison with other techniques

Comparison with other multilabeling approaches

Our approach allows the mapping of multiple projection neuron types using antibodies against their tracers. We were able to identify 3 of these pairs (Lucifer Yellow, Alexa Fluor 488, and Texas Red dextran and corresponding antibodies), and hopefully further exploration of more tracers will allow to extend the repertoire. We suggest that also non-fluorescent tracers should be tested. For example, dextrans could be labeled with tags which are resistant to osmification, possibly small molecules which have been shown to be detectable with postembedding immunohistochemistry (Anderson et al., 2011). Although these would have the disadvantage that they can only be detected only after the whole preparation has been performed, they could strongly increase the number of possible antibody-tracer combinations. More in general, it would be of high interest to identify molecules that better resist osmification, or to produce antibodies against osmified versions of the molecules. This flexibility resulting from multiple correlative markers is useful when complex pathways have to be investigated, and presents an advantage over classical EM tracers, which require an
electron-dense substrate usually achieved by HRP-catalyzed polymerization of a chromogen and heavy-metal intensification. As introduced in chapter 1.3, only few studies have used 3 electron-dense tracers simultaneously, and the developing reaction needed resulted in a degradation of tissue ultrastructure (Anderson, Karle, and Reiner, 1994). For this reason, most of the studies only used 1 or 2 tracers, limiting the different subsets of neurons which could be investigated and reconstructed. Using these traditional EM methods, it would not therefore be possible to trace pathways composed of multiple neuron populations, for example to trace HVC-projecting Uva neurons and to distinguish whether synapses are made onto neurons projecting to RA or Area X, or to HVC interneurons (which could be identified with immunohistochemistry).

**Comparison with other correlative approaches**

Other correlative tissue preparation approaches exist, as we have introduced in chapter 1.5. Photooxidation combines fluorescence LM and EM, by using fluorescent dyes to oxidize diaminobenzidine into an electron-dense osmiophilic polymer (Maranto, 1982). The advantage of photooxidation is that a direct and unambiguous correlation of a structure in the LM and EM is possible. This technique has however some drawbacks, in particular that the volume of tissue which can be photoconverted is limited and that, due to photoconversion times differing among fluorophores, the technique has to our knowledge never been applied to photoconvert different fluorophores simultaneously in the same tissue. Moreover, even if this would be possible, a method should be developed to convert different colors into substrates distinguishable in the EM.

Quantum dots have also been shown to be a valid correlative marker, which emit fluorescence of different wavelength depending on their size, which is in turn distinguishable in the EM (chapter 1.5.2). So far, however, they have only been used conjugated to antibodies, and not as tracers. We conducted some preliminary experiments, in which we injected a previously mixed solution of quantum dots-streptavidin and BDA (data not shown). Unfortunately, the quantum dots did not diffuse into the neurons injected, but rather formed some cluster of fluorescence around the injection site. It would be of high interest to develop a quantum dot form that can be used as tracer, perhaps conjugated to dextrans or coated in a way that they can diffuse into neurons without clustering or interacting with endogenous structures.

Cryosectioning has also been used in correlative microscopy, for example combined with immunohistochemical methods thanks to increased antigenicity preservation (chapter 1.5.3). We have also explored the idea of detecting neural tracers directly in ultrathin cryosections, because in principle fluorescence quenching
steps are omitted in the preparation (the sections only fixed with paraformaldehyde, infiltrated with sucrose, and frozen, see chapter 3.3). Although fluorescence can be indeed preserved, cryosectioning has two major drawbacks for neural circuit reconstruction: it is almost impossible to collect large numbers of serial sections, due to section instability, and the membrane contrast in the EM is low, thus making segmentation difficult.

The ideal approach to correlative microscopy would be direct correlation of fluorescence LM and EM data. It would be ideal to first image with fluorescence LM the tissue, and then prepare it with the best possibly quality for EM, without needing to compromise the preparation to preserve fluorescence, photoconvert it, or to preserve antigenicity. After imaging, the two datasets could be overlaid. This approach has been already used to identify neuron somata, imaged in vivo with LM, in SBEM and ssTEM volumes (chapter 1.5.3).

In our experience biological tissue undergoes strong morphological transformations (in particular shrinkage) during preparation for EM, both during heavy metal treatment, dehydration and embedding, and during ultrathin sectioning. Whereas using endogenous features it is possible to correlate cell bodies, to our knowledge correlation of large numbers of smaller (unlabeled) features such as axons or dendrites in a non-homogeneously distorted tissue is still a task that is computationally unresolved. We think that our correlative array tomography approach will help to develop tools to automatically register LM (most commonly confocal LM) and EM volumes. Correlative array tomography will provide local correspondences between pre-embedding LM and EM, which can be used to check the correctness of the alignment. These correspondences consist of different tags whose identity has to match with the multicolor tracers in the LM (based on the color in the confocal and the antibody on the ultrathin sections), and is localizing with specific structures in perfectly-aligned EM micrographs (the immunostaining is done on the same ultrathin sections imaged in the EM).

**Comparison with dense reconstruction EM-based methods**

Compared to block-face imaging techniques or approaches relying on dense EM reconstruction, our approach has the advantage that the time-consuming operation of EM imaging and tracing can be guided by LM information, which can be acquired much faster. For example, by comparing consecutive sections and setting an appropriate threshold, it should be possible to define a list of regions containing a neuron of a certain class with a high probability, and limit the time-consuming EM imaging to these regions. Moreover, by combining information from multiple immunostaining and fluorescence channels, it should be
possible to further optimize EM imaging by restricting it to certain regions, for example based on the presence of processes of two different neuron populations (labeled with two different tracers) within a certain radius or, if a reliable antibody against synaptic molecules is found, on the presence of a putative synapse close to processes of different classes. In addition, until a technique is developed which allows to image at synaptic resolution an entire brain, or until different tracers distinguishable in the EM are developed, our approach has the advantage over EM-based techniques that it allows to capture in the same volume the identity of neurons projecting to different brain regions located far apart, and allows to reconstruct only local portions of neurons in a targeted way.

2.3.5 Reconstruction of identified neurons in the songbird brain

We used to methods to characterize location of projection neuron somata in HVC, synapses made by Uva_{HVC} neurons in HVC, and synapses on HVC_{RA} neurons. In the case of projection neuron somata, we noticed in the EM how neuron somata tend to cluster in HVC, similar to what has been reported in the canary (Burd and Nottebohm, 1985). Using two different fluorescence channels registered with EM imagery, we were able to observe a case in which two HVC_{RA} and HVC_{X} somata were in direct contact. It would be interesting to further investigate whether neurons are coupled by gap junctions, which we were not able to detect, as in the canary and whether these occur between neurons of different populations (HVC_{RA} and HVC_{X}) or only between neurons of one population, whether these somata clusters have a functional role, e.g. by comparing with in vivo imaging the activity of different neurons within one cluster, and whether these clusters are important for development and integration of new cells, as neurogenesis can occur in the adult zebra finch in HVC (Scharff et al., 2000).

We also characterized synapses made by Uva_{HVC} neurons in HVC. We found two types of synapses based on the morphology of the presynaptic bouton. In some cases, the synapses were part of a large bouton containing several mitochondria and other large vesicle-like intracellular organelles. These boutons only made a single synapse, either with large postsynaptic partners receiving multiple synapses, or with smaller partners receiving only a synapse. In another case, we found the synapse of the Uva_{HVC} neuron to be on an axon whose diameter in the proximity of the synapse was not larger than in more distal parts, whereas the postsynaptic partner tended to be larger around the synapse. Single spikes in Uva_{HVC} neurons, which predominate in their activity, have been shown to suppress spontaneous and auditory-evoked bursts in HVC and RA neurons, whereas Uva_{HVC} bursts are excitatory, preceding bursts in HVC interneurons and RA
neurons (Hahnloser et al., 2008). This suppressive effect of Uva on HVC bursts through single spikes in Uva\textsubscript{HVC} neurons seems to dominate in the interaction of Uva and HVC, as shown by the fact that pharmacological inactivation of Uva leads to increased cerebral bursting, and the suppressive action of these spikes seems to be associated with the behavioral state of the animal. It is unclear how single spikes and bursts in Uva\textsubscript{HVC} mediate different effects on HVC, and it also unclear which neurotransmitter type is used by Uva\textsubscript{HVC} neurons. In all synapses we reconstructed, the contact was thick and strongly electron-dense, suggestive of an asymmetric, and thus probably excitatory, synapse, although the shape of the vesicles could not be unambiguously clarified in all cases (round vesicles were well visible in some cases, but not always). It is possible that single Uva\textsubscript{HVC} neurons make synaptic contacts with multiple neuron types, e.g. with both interneurons and projection neurons within HVC, and that the difference in morphology of the boutons (and possibly of the synaptic vesicles) reflects the identity of the postsynaptic partner and a different activation strategy, for example with synapses on interneurons activable already by single spikes and synapses on projection neurons only by bursts of spikes. In the present thesis, we reconstructed only few Uva\textsubscript{HVC} synapses, and we were not able to identify the postsynaptic partner because we only performed a single immunostaining against Uva\textsubscript{HVC} neurons (the other channel of the light micrographs was direct fluorescence, which was only detectable in the soma and large processes). Our correlative array tomography approach, however, should allow to identify the postsynaptic dendrite, for example by following the dendrite in electron micrograph up to the soma (if using only one immunostaining round for one tracer and direct fluorescence for the other is used) or if a second signal is directly present in the postsynaptic neuron (by combining two or more anti-dye antibodies).

Moreover, we characterized with our correlative array tomography approach two synapses on the proximal part of an HVC\textsubscript{RA} dendrite. Both synapses were symmetric and part of large boutons containing multiple synaptic contacts with other neurons as well. The morphology of the synapses, symmetric, hints at an inhibitory synapse type, and therefore the presynaptic bouton may belong to an interneuron. If this is true, this synaptic complex may correspond to swellings (varicosities) noticed on inhibitory neurons in HVC (Mooney, 2000).

2.3.6 Conclusion

We think that our method, rather than provide the full connection matrix between all individual neurons in a brain region, will be useful to characterize and
statistically quantify connections between different projection neurons. The overall goal that motivated this work is to learn more about the wiring diagram of a sensorimotor network which underlies a very complex behavior such as song learning and production. We hope that our method will help us discover how neural signals are routed into and out of different brain regions, for example HVC, by providing quantitative information about strength and number of connections made by different neuron types onto the same or other neuron types, and by that help formulating better models of how the song-control network in the songbird functions.

2.4 Methods

This part describes the general preparation protocol, deviations from this protocol and location of the injections for individual birds are indicated in section 2.2.

2.4.1 Tracers injection

The bird was first anesthetized and placed in stereotaxic apparatus. Anesthesia was induced by 3-4% isofluorane in O₂ delivered with a mask placed over the beak, subsequently maintained by a lighter concentration (1.5%-2%) delivered with a tube inserted in the beak once the animal was in the stereotaxic apparatus. Feathers were removed from the skull, lidocain gel was applied to the skin and this was opened 10 min later by a single incision from the base of the beak to the dorsal attachment of dorsal muscles of the neck. The angle of the head was adjusted to 65° using a metal stick placed in a ridge formed by the skull along the midline close to the beak. The outer and middle bone layers of the skull were removed with a drill in a 1 millimeter diameter region above lambda. Lambda was then used as a reference point to locate the regions of interest. To access these, a hole in the skull was drilled so that the inner bone layer was so thin to be breakable with forceps. After removing this layer, a small hole in the dura mater was made by moving its fibers with a tungsten wire. Through this hole a pipette previously filled with a fluorescent tracer was inserted into the brain. Pipettes were mostly pulled from borosilicate glass (with filament) capillaries with an inner diameter of 0.94 mm and an outer diameter of 1.2 mm. For injections, pipettes were connected to a Picospritzer Microinjector (General Valve corporation) set to a pressure of 30 to 50 psi and a duration of 30 to 60 ms, with pressure pulses repeated many times depending on desired injection size, generally around 0.5 µl of different conjugated dextrans, judged by looking at the level of the tracer in the pipette. After each injection, the pipette
was slowly retracted, controlling that the tracer did not flow back through the pipette tract. At the end of all injections, the skin over the skull was closed again, glued with a topical tissue adhesive (Gluture), and the lidocain gel was again applied to the skin. After few minutes the anesthesia was discontinued, the bird was removed from the stereotaxic apparatus and placed in a single cage for recovery.

2.4.2 Perfusion, sectioning, imaging

After a survival time of around 5 days, the bird was given a lethal intraperitoneal dose of pentobarbital, usually around 50 µl. As soon as the animal was deeply anesthetized, it was placed on a styrofoam board and its wings and legs spread and fixed with needles. The thorax was then opened to have access to the heart. After injecting 20 µl heparin into the left ventricle, a small incision (1-2 mm in length) was made in the right atrium. The bird was then perfused with 5 ml 0.9% NaCl, followed by 300 ml of 2% paraformaldehyde and 0.075% glutaraldehyde in phosphate buffer (0.1 M, pH 7.4, PB). During the first minute of the perfusion, high pressure was manually applied to the perfusing solution, while later the bottle containing the fixative was raised of around 1 meter relative to the animal position.

After perfusing, the head was detached from the bird and the skull was carefully opened. The brain was removed and placed for 1 h in the same fixative solution using the perfusion. The brain was then washed with PB and 60-µm-thick sagittal sections were cut in 4 °C PB with a vibratome. The sections were stored in 24-cell wells at 4°C until further processing. In some cases, the brain was embedded in 1-2% agar to have more stability while cutting. The sections were then places on glass slides and observed in a wide-field fluorescence microscope (Olympus BX61) to localize the area of interest. Overview images were also acquired with fluorescence and transmitted light. These images are particularly useful after embedding, because fluorescence is not visible anymore and localization of the region of interest is facilitated by comparing where the region was relative to blood vessels, which are also well visible in the embedded tissue.

Additionally, in some cases sections containing HVC were imaged with a confocal laser scanning microscope (Leica SP5). Typically, images were acquired with 63x, NA 1.3 glycerol objective using sequential scans for different fluorophores, with excitation lasers and detection spectra calibrated to minimize channel crosstalk. Confocal image stacks were analyzed using Bitplane Imaris 3.1 software.
2.4.3 Tissue preparation

Sections were transferred to glass vials for further processing, to which solutions were added and removed with a glass pipette (during osmification) or plastic pipette (during dehydration). They were washed in cacodylate buffer (0.1M, pH 7.4), followed by post-fixation and staining for 40 min in 1.5% potassium ferrocyanide and 1% OsO₄ in cacodylate buffer (0.1 M, pH 7.4). They were then incubated for 1 h in 1% OsO₄ (also in cacodylate buffer (0.1 M, pH 7.4)) and for 1 h in 1% uranyl acetate in distilled water (this staining protocol was adopted from (Knott et al., 2008)). Sections were then dehydrated in a series of ethanol dilutions, starting with 10 min 50% ethanol in distilled water, followed by 10 min 70% ethanol, 10 min 90% ethanol, 10 min 90% ethanol, 10 min 95% ethanol, twice 15 min 100% ethanol, and finally twice 15 min propylene oxide for complete dehydration. After dehydration, the sections were embedded in Durcupan ACM resin (10 g component A/M, 10 g component B, 0.3 g component C, 0.2 g component D). Sections were put overnight in a small container filled with resin and on the following day transferred between two Aclar sheets, in turn sandwiched between two glass slides with a small weight on the upper one. Sections were then cured for 48 h at 52°C.

Once cured, the glass slides were removed, as well as one of the two Aclar sheets. Sections were observed with the light microscope with transmitted light and, using the reference images previously acquired, the region of interest (typically HVC) was localized. A small block of tissue (< 2 mm side length) containing the region of interest was manually resected with a breakable razor blade held in a blade holder and attached with a super glue to a blank resin block. The block was then transferred to an ultramicrotome for further trimming and ultrathin sectioning. Using a glass knife, the edges were trimmed so that the surface of the block was less than 1 mm². The block was repeatedly observed with a light microscope and compared to the reference image to optimize the position and amount of trimming. After trimming, the surface of the block was aligned to the diamond knife to start ultrathin sectioning. Sections were collected on different substrates depending on the experiment and electron microscope used. For the first experiments in which sections were imaged in a TEM, these were placed on pioloform-coated 2 x 1 mm nickel grids (the grids were coated in-house with a pioloform film of a thickness around 80-100 nm). For later experiments, imaging was done in SEM, and sections were placed either on ITO-coated glass slides, or in most cases on silicon wafers. In this case, a custom-made diamond knife inserted in a 2.5 cm x 6.5 cm boat, so that complete glass slides or big silicon wafers could be used, allowing for collection of long series of consecutive sections. Ultrathin sections were sectioned at a thickness in the range of 60-90
nm. In the case of long ribbons of sections, care was taken during trimming to have perfectly parallel block edges, which lead to sticking of consecutive sections with each other.

2.4.4 Light microscopy and immunostaining

Ultrathin sections were observed in the light microscope (Olympus BX61) for detection of direct fluorescence. Sections on pioloform-coated grids were imaged with an air lens (e.g. 40x, NA 0.6), because covering with glass for imaging with oil lenses was not possible due to the fragility of the pioloform. Sections on ITO-coated glass and silicon wafer were in contrast covered with a mounting medium (SlowFade Gold with DAPI, Invitrogen) and a cover glass, and imaged with an oil lens with high numerical aperture (e.g. 60x, NA 1.42). The short edges of the cover glass were attached to the slide using an epoxy glue, to avoid sliding of the cover glass during image acquisition when using oil lenses. Exposure time of at least 1 s were necessary due to the low signal intensity. After imaging, the cover glass was removed and the sections were washed in distilled H$_2$O. Image acquisition was automated using the "Experiment Manager" package of the CellR software. To increase the signal and localize tracer whose fluorescence was lost during the preparation, immunostaining on the ultrathin sections was performed. Sections were treated for 10 min with 1% periodic acid in the case of grids for TEM, or for 10 min with 1% sodium metaperiodate in the case of sections for SEM. Sodium metaperiodate is believed to reverse OsO$_4$ fixation and, similar to periodic acid, improves antibody access in epoxy resins (Stirling and Graff, 1995). Moreover, sodium metaperiodate has been shown to restore immunolabeling without altering structure (Bendayan and Zollinger, 1983). Sections were then washed with distilled H$_2$O, by either dipping the grids 15 times in water-filled glass vials, or by letting water flow over the sections on the glass slides and silicon wafers. Sections were washed two times for 10 min in phosphate-buffered saline (TPBS, pH 7.4), then pre-blocked with either normal goat serum (NGS) or acetylated bovine serum albumine (BSA), depending in particular on the microscopy modality. For TEM, sections were treated for 30 min with 5% goat serum in TPBS and for 10 min with 1% goat serum in TPBS, whereas for SEM sections were treated for 30 min with 1% BSA in TPBS. TEM grids were then inverted on small droplets of diluted primary antibody for 1.5 h (e.g. 1:50, rabbit anti-Lucifer yellow), whereas sections for SEM were incubated for 1.5 h with same concentration of diluted primary antibodies in 1% BSA in TPBS. Sections were washed 4 times with TPBS, followed by incubation in secondary antibodies for 1.5 h, typically 1:50, Alexa Fluor 594 or 647 goat anti-rabbit IgG. Finally they were washed twice for 5 min with TPBS and twice in
distilled H$_2$O. As before for direct fluorescence, TEM grids were imaged with an air lens, whereas SEM sections were covered with a mounting medium and a cover glass and imaged with an oil lens.

2.4.5 Elution

For immunostaining of multiple antigens with antibodies raised in the same animal species, the elution protocol introduced by (Micheva et al., 2010) was adopted. The glass slide was removed from the silicon wafer and the sections were washed with Tris. The sections were then incubated for 20 min with the elution solution, consisting of 0.2 M NaOH and 0.02% of SDS in distilled H$_2$O. They were then gently rinsed and incubated for 10 min with Tris twice, rinsed with distilled H$_2$O, and finally dried and stored at room temperature. Subsequent immunostainings were performed as in 2.4.4, except for the omission of the first step with period acid or sodium metaperiodate. We performed the elution with ultrathin sections on different substrates, including carbon-coated glass, indium tin oxide (ITO)-coated glass, and silicon wafer. Carbon-coated glass did not compatible with the elution, because the carbon coating detached due to the treatment with the elution solution. Sections on ITO-coated glass tended to suffer of damage during elution, with some sections breaking and detaching from the glass. Sections seemed in contrast to suffer only limited damage when on silicon wafer, with folds forming occasionally, but no detachment of the sections.

2.4.6 Electron microscopy

After imaging in the FLM, sections were poststained for electron microscopy. TEM sections and SEM sections which were not eluted were first they were stained for 3 min in 1% uranyl acetate and washed 3 times for 30 sec in distilled H$_2$O. They were then stained for 2 min in Reynold’s lead citrate and washed 3 times for 30 sec in distilled H$_2$O. SEM sections which underwent elution were in contrast stained for 1 min in 1% OsO$_4$, washed 3 times with distilled H$_2$O, stained for 30 min in 5% uranyl acetate, washed 3 times with distilled H$_2$O, and finally stained for 3 min with Reynold’s lead citrate and washed 3 times with distilled H$_2$O. TEM sections were imaged on a FEI Morgagni 268 microscope, whereas SEM images were acquired with Zeiss NVision 40 with ESB detector and voltages of 1.2-1.5 kV. For SEM, it is important to optimize voltage so that the beam reaches the biological tissue, because the latter is covered by the blocking solution and other chemicals used for immunostaining.
2.4.7 Image processing

Image processing was done with ImageJ, TrakEM2 (an ImageJ package) and Adobe Photoshop CS3. First, electron micrographs were imported in TrakEM2 and aligned linearly, followed by light micrographs. Light micrographs were often acquired in 3 x 3 arrays for each channel in an imaging sessions, therefore with the images of different channels spatially overlapping, and multiple imaging sessions for each section, with the images in this case not overlapping, since the probe was not in the same position in the microscope in each session. Images of the channel of the first imaging session containing most features were first imported into TrakEM2 as a grid, since the approximate position of each image relative to the others is known. Alignment was further improved by phase-correlation or SIFT features of the overlapping regions. Image arrays of consecutive sections were then registered with each other using SIFT-based affine transformations. After complete registration of one channel, affine transforms of this channel were applied to the corresponding patches of the other channels acquired within the same imaging session. Subsequently the first channel of the second session was imported, images of the first layer were aligned with each other and with the corresponding layer of the first imaging session, and alignment for the consecutive sections was semi-automated by combining the affine transforms applied during the first session with the rotation and translation applied to align the two sessions. The same procedure was applied for all imaging sessions.

In the case of reconstructed neurons, two TrakEM2 projects were prepared in parallel. One was prepared as described as above (with linear transformations only), and was used for LM-EM correlation. The other project was composed of electron micrographs only which were aligned with elastic spring-mesh registration, and was used to trace and reconstruct over multiple sections neurons identified with fluorescence signal in the first project. Correlative images were prepared either in Photoshop only, or first in TrakEM2 and then in Photoshop (in this case light micrographs enlarged in TrakEM2 were blurred in Photoshop). In Photoshop, the light micrographs were colored and overlaid to the electron micrographs.
Figure 2.26: Schematic drawing of the preparation protocol. The preparation includes in vivo injection of tracers, embedding of the tissue for electron microscopy, immunostaining of the ultrathin sections, light and electron microscopy of the sections. See text for detailed explanation of the various steps.
Chapter 3

Osmium tetroxide-free tissue preparation

3.1 Osmium tetroxide- and uranyl acetate-free preparation

Preliminary experiments showed that a classic tissue preparation protocol, including osmium tetroxide and uranyl acetate, produces excellent ultrastructural quality with good membrane preservation for electron microscopy, yet some steps drastically reduce fluorescence levels of the tracers that are used for cell labeling. OsO$_4$ is probably the most critical chemical that is used in the protocol. It has the highest oxidation state achieved by a transition element (+8) and reacts with nucleophiles (e.g. amines) and in particular alkenes. On the one hand this results in strong fixation of the tissue - therefore preservation of the ultrastructure (membrane and synaptic vesicles) - with accumulation of the chemical in the membrane (and thus staining of the membranes for EM); on the other side, however, its oxidative and proteolytic properties lead to a drastic reduction of the fluorescence, at least when OsO$_4$ is used at RT. A series of initial experiments have been conducted avoiding the use of osmium tetroxide and uranyl acetate (UAc). Their omission was compensated by a long post-staining with uranyl acetate (20 min) and lead citrate (2 – 3 min). The tissue was embedded in LR White resin, which was chemically cured, to avoid prolonged heating of the probes (Table 3.1).

The preparation resulted in poor membrane preservation (Fig 3.2 and 3.3). Moreover, the tissue showed signs of extraction, probably due to poor fixation prior to dehydration and embedding. An additional experiment was conducted, in which the tissue was only partially dehydrated (up to 95% ethanol) and chem-
<table>
<thead>
<tr>
<th>Step</th>
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<th>Temp</th>
</tr>
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<tr>
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<td>RT</td>
</tr>
<tr>
<td>4% paraformaldehyde</td>
<td>2 h</td>
<td>4 °C</td>
</tr>
<tr>
<td>20% sucrose</td>
<td>6 h</td>
<td>RT</td>
</tr>
<tr>
<td>30% sucrose</td>
<td>12 h</td>
<td>RT</td>
</tr>
<tr>
<td>80 µm slicing</td>
<td></td>
<td>RT</td>
</tr>
<tr>
<td>50% ethanol</td>
<td>10 min</td>
<td>4 °C</td>
</tr>
<tr>
<td>70% ethanol</td>
<td>10 min</td>
<td>4 °C</td>
</tr>
<tr>
<td>90% ethanol</td>
<td>15 min</td>
<td>4 °C</td>
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<td>100% ethanol</td>
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</tr>
<tr>
<td>LR White infiltration</td>
<td>12 h</td>
<td>4 °C</td>
</tr>
<tr>
<td>Resin curing with chemical accelerator</td>
<td></td>
<td>RT</td>
</tr>
<tr>
<td>80 nm slicing</td>
<td></td>
<td>RT</td>
</tr>
<tr>
<td>Uranyl acetate poststaining</td>
<td>20 min</td>
<td>RT</td>
</tr>
<tr>
<td>Lead citrate poststaining</td>
<td>2-3 min</td>
<td>RT</td>
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</tbody>
</table>

Figure 3.1: Preparation protocol without OsO₄ and UAc (animal b16p).

Figure 3.2: Electron micrograph of tissue prepared without OsO₄ and uranyl acetate, showing poor tissue preservation.
ical curing was done on ice, to avoid overheating of the probe which could be caused by the heat generated during the rapid polymerization process (Table 3.4). These changes did not lead to an improvement in the quality of the tissue (Fig 3.5).

We then tried to improve tissue ultrastructure by increasing the initial fixation during the perfusion, in particular using a high concentration of glutaraldehyde (Table 3.6). We injected an animal with Alexa Fluor 488 dextran in HVC and analyzed regions of tissue containing retrogradely labeled cells (Ni and Uva). We embedded the tissue in LR White and tested different polymerization processes, chemical and thermal, and found better results with chemical curing. High glutaraldehyde concentration led to an improvement of general tissue morphology (Fig. 3.7), detailed observation, however, revealed that membrane were still not preserved (Fig. 3.8). Fluorescence was still detectable in ultrathin sections, concentrated in vesicles (Fig. 3.9). We tried to recover signal by using anti-Alexa Fluor 488 antibodies followed by fluorescently-labeled secondary antibodies and acquired images of the same cell in both LM and EM. Immunolabeling allowed us to delineate the contour of the soma and presumably some processes, although we could not test their identity in the EM image because of poor ultrastructure preservation (Fig. 3.10).
<table>
<thead>
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<tr>
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<td></td>
<td></td>
</tr>
<tr>
<td>4% paraformaldehyde</td>
<td>2 h</td>
<td>4 °C</td>
</tr>
<tr>
<td>20% sucrose</td>
<td>6 h</td>
<td>RT</td>
</tr>
<tr>
<td>30% sucrose</td>
<td>12 h</td>
<td>RT</td>
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<tr>
<td>80 µm slicing</td>
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<td>RT</td>
</tr>
<tr>
<td>50% ethanol</td>
<td>10 min</td>
<td>4 °C</td>
</tr>
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<td>70% ethanol</td>
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<td>4 °C</td>
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<tr>
<td>95% ethanol</td>
<td>15 min</td>
<td>4 °C</td>
</tr>
<tr>
<td>LR White infiltration</td>
<td>12 h</td>
<td>4 °C</td>
</tr>
<tr>
<td>Resin curing with chemical accelerator</td>
<td></td>
<td>on ice</td>
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<tr>
<td>80 nm slicing</td>
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<td>RT</td>
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<tr>
<td>Uranyl acetate poststaining</td>
<td>20 min</td>
<td>RT</td>
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<tr>
<td>Lead citrate poststaining</td>
<td>2-3 min</td>
<td>RT</td>
</tr>
</tbody>
</table>

Figure 3.4: Preparation protocol without OsO$_4$ and UAc, partial dehydration and curing on ice (animal green9).

Figure 3.5: Electron micrograph of tissue prepared without OsO$_4$ and uranyl acetate, and with partial dehydration and curing on ice. Preservation of tissue ultrastructure is insufficient.
<table>
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<th>Step</th>
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<tr>
<td>2.5% glutaraldehyde</td>
<td></td>
<td></td>
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<tr>
<td>Perfusion with 2% paraformaldehyde and</td>
<td>1 h 30 min</td>
<td>4 °C</td>
</tr>
<tr>
<td>2.5% glutaraldehyde</td>
<td></td>
<td></td>
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<tr>
<td>80 µm slicing</td>
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<td>RT</td>
</tr>
<tr>
<td>50% ethanol</td>
<td>10 min</td>
<td>on ice</td>
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<tr>
<td>70% ethanol</td>
<td>10 min</td>
<td>on ice</td>
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<tr>
<td>90% ethanol</td>
<td>15 min</td>
<td>on ice</td>
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<tr>
<td>95% ethanol</td>
<td>15 min</td>
<td>on ice</td>
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<tr>
<td>Incubation in 2:1 ethanol:LR White</td>
<td>1 h 30 min</td>
<td>on ice</td>
</tr>
<tr>
<td>Incubation in 1:2 ethanol:LR White</td>
<td>1 h 30 min</td>
<td>on ice</td>
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<tr>
<td>100% LR White infiltration</td>
<td>overnight</td>
<td>4 °C</td>
</tr>
<tr>
<td>LR White change</td>
<td></td>
<td>on ice</td>
</tr>
<tr>
<td>Resin curing with chemical accelerator /</td>
<td>- / 72h</td>
<td>on ice / 60 °C</td>
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<tr>
<td>thermally</td>
<td></td>
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<td>80 nm slicing</td>
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</tr>
<tr>
<td>4% OsO₄ vapour poststaining</td>
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</tr>
<tr>
<td>2% uranyl acetate poststaining</td>
<td>5 min</td>
<td>RT</td>
</tr>
<tr>
<td>Lead citrate poststaining</td>
<td>5 min</td>
<td>RT</td>
</tr>
</tbody>
</table>

**Figure 3.6:** Preparation protocol without OsO₄ and UAc, but high glutaraldehyde concentration (animal r1y20).

**Figure 3.7:** Tissue prepared with higher glutaraldehyde concentration (2.5%) is better preserved, but quality is not sufficient (animal r1y20).
Figure 3.8: Higher magnification image of the tissue in fig. 3.8 (animal r1y20).

Figure 3.9: Fluorescence in ultrathin sections of tissue prepared with high glutaraldehyde concentration (animal r1y20).
3.2 Tannic acid as a substitute for osmium tetroxide

Tannic acid is a mixture of polyglycol anions which binds to a broad variety of tissue macromolecules, including proteins, saturated phospholipids, and complex carbohydrates (Berryman et al., 1992). Application of tannic acid on tissue previously fixed by aldehydes and OsO₄ has been shown to induce general high contrast with fine delineation of membranes. Tannic acid has a complex effect on fixed tissue, acting primarily as a mordant for heavy metals between osmium-treated structures and lead but also stabilizing some tissue components against extraction and deterioration during dehydration and resin embedding (Simionescu and Simionescu, 1976). Additional experiments have been performed to investigate the effects of tannic acid on antigenicity and membrane contrast (Berryman et al., 1992). The authors tested the effects of tannic acid on antigenicity and morphology of epithelial cells of rat liver and intestine in LR Gold acrylic-resin embedded tissue. Even in the absence of OsO₄, tannic acid consistently enhanced membrane contrast when used either as part of the primary fixative or before UAc treatment, showing that OsO₄ is not a prerequisite for obtaining high levels of membrane contrast. This effect was possibly due to tannic acid binding sufficient UAc as well as additional heavy metals used to enhance contrast on ultrathin sections. Moreover, the authors found that after treatment with low concentrations of tannic acid (<0.25%), acceptable levels of antigenicity were retained for several membrane antigens tested with-
out an increase in background immunostaining, although tannic acid resulted in up to 79% loss of antigenicity relative to tissue treated with UAc alone. An additional study introduced additional steps, enabling structural preservation while retaining antigenicity in epoxy-embedded brain tissue (Phend, Rustioni, and Weinberg, 1995). The protocol included perfusion with 2.5% glutaraldehyde and 1% paraformaldehyde, processing of the sections on ice, incubation with tannic acid followed by UAc, and treatment of the sections with the organic compound p-Phenylenediamine (PPD) and the heavy metal salt platinum chloride (PC). PPD, in particular, known to preserve lipids, led to unexpected antigenicity enhancement, possibly through a removal of excess UAc present in the tissue. These results showing that tannic acid may be used as a substitute of OsO₄, led us to test the possibility that if antigenicity was preserved, also fluorescence may be affected less severely than in OsO₄-treated tissue. We started to investigate the use of tannic acid by using it alone before dehydration, when OsO₄ is normally used, at different concentrations and incubation times (0.1% vs. 1%, 20 min vs. 1 h), with embedding of the tissue in LR White (Table 3.11). Quality of tissue prepared with tannic acid (Fig 3.12, 1% tannic acid for 20 min) was similar to control tissue prepared in the same way but without tannic acid (Fig 3.13), showing signs of tissue extraction and bad membrane preservation and contrast for all concentrations and times tested. The lack of positive effect of tannic acid may be due to fact that the mechanism by which it increases membrane contrast probably results from its action as a mordant for heavy metals, such as UAc (Berryman et al., 1992), and therefore tannic acid alone does not help preserving the tissue.

Figure 3.12: Tissue prepared with incubation in 1% tannic acid for 20 min.
<table>
<thead>
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<th>Temp</th>
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<tr>
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<tr>
<td>0.3% glutaraldehyde</td>
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<td>Postfixation in 4% paraformaldehyde and</td>
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<td>4 °C</td>
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<tr>
<td>0.3% glutaraldehyde</td>
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<td></td>
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<tr>
<td>80 µm slicing</td>
<td></td>
<td>RT</td>
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<tr>
<td>Incubation in tannic acid</td>
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<td>10 min</td>
<td>RT</td>
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<td>10 min</td>
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<tr>
<td>90% ethanol</td>
<td>15 min</td>
<td>RT</td>
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<tr>
<td>95% ethanol</td>
<td>15 min</td>
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<tr>
<td>Incubation in 2:1 ethanol:LR White</td>
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<td>Incubation in 1:2 ethanol:LR White</td>
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<tr>
<td>100% LR White infiltration</td>
<td>2 h</td>
<td>4 °C</td>
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<tr>
<td>Resin curing with chemical accelerator or</td>
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<td>on ice / 60 °C</td>
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<td>thermally</td>
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<tr>
<td>80 nm slicing</td>
<td></td>
<td>RT</td>
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</table>

**Figure 3.11**: Preparation protocol with tannic acid (animal r4r15).

**Figure 3.13**: Control tissue prepared the same way as in Fig 3.12 except for the omission of tannic acid.
We therefore conducted additional experiments with a protocol more closely following the one proposed by Phend et al., thus including tannic acid, UAc, PC and PPD, with the main differences of using a weaker perfusion fixative and LR White instead of Epon resin. Tissue prepared according to the protocol in table 3.14 showed a better morphological preservation, with good staining of synaptic densities, but membranes, although partially preserved, were hardly visible due to lack of specific staining on them (Fig. 3.15 and 3.16).

<table>
<thead>
<tr>
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<tr>
<td>Postfixation in 4% paraformaldehyde and 0.3% glutaraldehyde</td>
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<td>4 °C</td>
</tr>
<tr>
<td>80 µm slicing</td>
<td></td>
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</tr>
<tr>
<td>Incubation in 1% tannic acid</td>
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<td>on ice</td>
</tr>
<tr>
<td>Rinse in maleate buffer</td>
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<td>on ice</td>
</tr>
<tr>
<td>Incubation in 1% UAc</td>
<td>40 min</td>
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</tr>
<tr>
<td>Rinse in maleate buffer</td>
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</tr>
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<td>Incubation in 0.5% platinum chloride</td>
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</tr>
<tr>
<td>Rinse in maleate buffer</td>
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<td>on ice</td>
</tr>
<tr>
<td>50% ethanol</td>
<td>5 min</td>
<td>on ice</td>
</tr>
<tr>
<td>70% ethanol</td>
<td>5 min</td>
<td>on ice</td>
</tr>
<tr>
<td>Incubation in 1% PPD in 70% ethanol</td>
<td>15 min</td>
<td>on ice</td>
</tr>
<tr>
<td>Rinse 70% ethanol</td>
<td>2x</td>
<td>on ice</td>
</tr>
<tr>
<td>80% ethanol</td>
<td>5 min</td>
<td>on ice</td>
</tr>
<tr>
<td>95% ethanol</td>
<td>5 min</td>
<td>on ice</td>
</tr>
<tr>
<td>Incubation in 2:1 ethanol:LR White</td>
<td>2 h</td>
<td>RT</td>
</tr>
<tr>
<td>Incubation in 1:2 ethanol:LR White</td>
<td>6 h</td>
<td>RT</td>
</tr>
<tr>
<td>100% LR White infiltration</td>
<td>2 h</td>
<td>4 °C</td>
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<tr>
<td>Resin curing with chemical accelerator</td>
<td></td>
<td>on ice</td>
</tr>
<tr>
<td>80 nm slicing</td>
<td></td>
<td>RT</td>
</tr>
</tbody>
</table>

Figure 3.14: Preparation protocol with tannic acid similar to (Phend et al., 1995) (animal y4r1).

Levels of fluorescence of tracers injected in the tissue were also analyzed, to test whether the preparation had a detrimental effect on fluorophores. We therefore injected an animal (p17p) with Alexa Fluor 488 dextran in HVC and analyzed fluorescence levels in the retrogradely-labeled nucleus NIf. Some sections were processed with the protocol in table 3.14 but omitting UAc, PC, and PPD (Fig. 3.17), some sections omitting only PC and PPD (Fig. 3.18), whereas other sections were prepared with the complete protocol (Fig. 3.19). A strong reduction in fluorescence was observed when the complete protocol was
Figure 3.15: Tissue prepared with tannic acid, UA, PC and PPD shows better preservation than tissue prepared with UA alone, but low membrane contrast.

Figure 3.16: Different micrograph of the same tissue as in Fig 3.15.
followed, compared to tissue prepared with tannic acid alone, with the main reduction observed already after UAc incubation.

These experiments with tannic acid resulted in insufficient ultrastructure quality (in particular membrane contrast) and at the same time a strong reduction in fluorescence. Because information lacking in the EM data, in particular good membrane delineation for tracing of neurons over multiple sections, was not compensated by the fluorescence signal, we did not further investigate in this direction. The decision to use LR White was motivated by the fact that the epoxy resin we were using for other experiments, Durcupan, was strongly autofluorescent when observed under a LM before ultrathin sectioning (data not shown). Subsequent experiments we have conducted, however, have shown that on the one hand Durcupan leads to better tissue preservation (in particular less extraction), and on the other its autofluorescence is not problematic when working with ultrathin sections. It could be therefore of interest to test again the protocol with an epoxy resin, to evaluate if it leads to better results.

Figure 3.17: Retrogradely-labeled NF is well visible in tissue prepared with tannic acid alone.

3.3 Tokuyasu cryosection method

An alternative method to resin embedding and sectioning for electron microscopy is Tokuyasu cryosectioning (Tokuyasu, 1973; Tokuyasu, 1986). This technique is of high interest for subcellular immunocytochemistry, because the light tissue preparation involved allows detection of some antigens which are not detectable
Figure 3.18: Embedding of the tissue with UAc in addition to tannic acid causes strong reduction of the fluorescence (image acquired with same illumination and exposure settings as fig 3.17.

Figure 3.19: Tissue embedded with PC and PPD in addition to tannic acid and UAc (image acquired with same illumination and exposure settings as fig 3.17.)
in resin-embedded tissue or more sensitive detection for others which are detectable with both methods (Webster, 1999). Unlike in classic preparation with resin embedding, in the Tokuyasu cryosectioning method freshly fixed tissue is frozen – in other words, the ice serves as an embedding material – and ultrathin sections are cut at low temperatures (<100 °C), therefore avoiding structural artifacts due to the OsO₄ and UAc treatment, dehydration and resin embedding. After thawing and drying at room temperature, sections can immunostained and imaged in the EM. The tissue is prepared as follows (see (Webster, 1999) for a detailed protocol). The tissue is chemically fixed, for example by perfusing the animal or immersing the tissue in a buffered aldehyde solution. Pieces of few cubic millimeters are cut and immersed for several hours in a 2.3 M sucrose solution. Sucrose acts as a cryoprotectant modifying the physical property of ice, so that the specimen is not damaged by the freezing. A small tissue piece is placed on a holder and frozen by immersion in liquid nitrogen, followed by the quick transfer to the cooled cryochamber of a specially-equipped ultramicrotome. After block trimming, ultrathin sections can be cut using a dry diamond knife. Sections are collected from the knife surface by picking them up on a small drop of methyl cellulose or sucrose suspended on a wire loop, transferred out of the cryochamber and placed onto a coated TEM grid. Sections can be immunolabeled, contrasted with UAc, and finally dried for EM imaging.

We conducted experiments to investigate if Tokuyasu cryosectioning is a method which is practicable for correlative microscopy and brain reconstruction. We injected an animal with Fluoro-Ruby dextran, perfused it with 2% PFA and 0.1% GA, cut 500 µm vibratome sections and cut out tissue blocks containing retrogradely labeled cells (Fig. 3.20). After sucrose infiltration and ultrathin sectioning, we imaged the sections with a LM. We found fluorescence signal distributed in small regions of size and shape similar to somata (Fig. 3.21). We imaged tissue prepared with the same method in the TEM, but ultrastructure quality was insufficient, with lack of contrast and tissue wash out (Fig. 3.22). Although we were able to improve tissue preservation in subsequent experiments, we were faced with a major limitation of Tokuyasu cryosectioning. Sections are extremely fragile, are susceptible to destruction due to electrostatic forces, and have to be collected individually. Collection of long series of sections, as it is necessary for brain reconstruction, is therefore unfeasible.
Figure 3.20: Tissue block with retrogradely labeled cells, to be prepared with the Tokuyasu cryosectioning method (animal r3r10).

Figure 3.21: Fluorescence in an ultrathin section from the tissue of figure 3.20, after preparation with the Tokuyasu cryosectioning method (animal r3r10).
Figure 3.22: EM quality of tissue prepared with the Tokuyasu cryosectioning method (animal r1a16).
Chapter 4

Viral vectors for neural labeling in songbird

Although fluorescent tracers are a valuable tool to trace projection neurons, the resulting labeling is not always as complete as wanted, for example with small processes and spines lacking fluorescent signal when retrogradely labeled. An alternative tool that has been applied to label neurons are viral vectors encoding a fluorescent protein. Several vector systems are available, based for example on lentiviruses, herpesviruses, or adeno-associated viruses. We tested the last one, because in mouse they have been shown to be retrogradely transported from the injected region along axons (Cearley et al., 2008), but have never been tested in the zebra finch.

4.1 Adeno-associated virus (AAV) vectors

4.1.1 Introduction

Adeno-associated virus (AAV) belongs to the Dependovirus genus of the Parvoviridae family (Buning et al., 2008). The name depends on the fact that productive infection of AAV depends on the co-infection of an unrelated helper virus, which provides factors necessary for active replication, such as adenovirus (Vasileva and Jessberger, 2005). AAV contains a single-stranded DNA genome of less than 4700 bases, enclosed in an icosahedral, non-enveloped capsid, and can be divided in three regions. The genome ends are 145 base-long inverted terminal repeats (ITR) forming a T shaped hairpin due to base pairing. (Wang, Faust, and Rabinowitz, 2010).

The injections were performed with M. Kirschmann.
AAVs are widely used as vector for gene delivery to the CNS, because they are non-pathogenic, result in long-term expression of the encoded gene (transduction has been demonstrated for a period longer than 1.5 years), and are able to transduce non-dividing cells (Xiao et al., 1997; Burger et al., 2004). Different serotypes are available and have been applied for gene transfer in the nervous system, mostly based on the genome of AAV2 cross-packaged with capsid proteins from other serotypes. We tested some of these serotypes in the zebra finch, an animal model for which there is still no literature regarding AAV vectors.

4.1.2 Results overview

We tested several AAV serotypes (all expressing GFP under the control of a CMV promoter), by injecting them in one hemisphere in RA and in the other in HVC (injections repeated in two animals, in more animals if the injection was at the wrong position), and observing after around 3 weeks GFP expression in particular in cells projecting to these two areas, as well as in axons of projection neurons whose soma resided in the two areas.

It was immediately apparent to us that retrograde labeling in HVC, when injecting in RA, was either present in few cells for each 80 µm section, or not at all. Because our motivation to use AAV was to improve retrograde labeling, which, with organic dyes coupled to dextrans, often resulted in limited labeling of the dendritic arbor, and with no serotypes we obtained this type of labeling in more than few cells, we decided to avoid a time-consuming analysis of the exact number of cells or axons labeled compared to the injection size and of the amount of signal present. Rather, we limited ourselves to a qualitative comparison of the different serotypes, which could be used as a starting point for further experiments which for example require retrograde labeling in few cells, or require delivery of a gene in a certain region without specific need of retrograde labeling.

We summarized the results in table 4.1, which we will discuss more in detail in the next sections.

4.1.3 Results detail: single-stranded AAVs

We first injected several AAV serotypes containing the DNA in its wild-type conformation, as a single-stranded molecule.

Serotype 2

Vectors based on AAV serotype 2 are the most common for delivery of genes to the central nervous system. rAAV2 have been shown to primarily transduce
<table>
<thead>
<tr>
<th>Serotype</th>
<th>Injection site</th>
<th>Anterograde</th>
<th>Retrograde</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>2/1</td>
<td>++</td>
<td>+</td>
<td>-</td>
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<tr>
<td>2/5</td>
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<td>+</td>
<td>-</td>
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<td>2/7</td>
<td>+</td>
<td>-</td>
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<tr>
<td>2/8</td>
<td>++</td>
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<td>-</td>
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<tr>
<td>2/9</td>
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<td>?</td>
</tr>
<tr>
<td>2/rh10</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>2/hu37</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>2/rh8</td>
<td>++</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>2/1 sc</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>2/5 sc</td>
<td>++</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>2/9 sc</td>
<td>+++</td>
<td>+</td>
<td>+ (?)</td>
</tr>
</tbody>
</table>

**Figure 4.1:** Summary table of different AAV serotypes tested. sc: self-complementary.
neurons, although not with equal effectiveness for all brain regions and with a relatively small volume of transduction (Taymans et al., 2007; Xiao et al., 1997; Burger et al., 2004).

We found rAAV2 to be the worse vector for transduction of neurons in songbird brain in the areas we tested. In both animals injected, we only found very few neurons (less than 5) labeled at the injection site in several sections we imaged.

**Serotype 2/1**

Recombinant AAV2/1 consists of a pseudotyped vector (a vector composed of the genome of one serotype inserted into the capsid of another serotype) in which the AAV2 genome is packaged into the AAV1 capsid. rAAV2/1 was found to transduce more cells and to result in detectable retrograde transport more frequently than rAAV2 in different regions of the rat CNS, including the hippocampus, the substantia nigra, the striatum and the spinal cord (Burger et al., 2004).

We found intermediate levels of transduction at the injection site compared to other serotypes (Fig. 4.2), with high expression of GFP in the transduced neurons (Fig. 4.3), but we did not find retrogradely labeled neurons.

![Light micrograph of the injection site, rAAV2/1 (animal o20r6). Field of view: 887 µm x 669 µm.](image)

**Serotype 2/5**

Similar to rAAV2/1, rAAV2/5 transduction distribution has been found to be wider than rAAV2 in the rat CNS (Burger et al., 2004).

We found rAAV2/5 to be the only single-stranded vector tested to result in
Figure 4.3: Light micrographs of cells transduced with rAAV2/1 at the injection site (animal o20r6). Field of view: 443 µm x 334 µm.

retrograde labeling of neurons. We found labeled neurons in HVC when injecting in RA (Fig. 4.4, in other sections more cells were visible), and also cells in NIf when injecting in HVC. Moreover, the number of transduced cells at the injection site was high and with strong labeling, and anterogradely labeled axons were well visible, for example in Area X after injection in HVC (Fig. 4.5). In an animal, we found a high number of retrogradely-transduced cells to express GFP, but at low levels (Fig. 4.6).

Serotype 2/6.2

rAAV6.2 showed an interesting expression pattern when injected into HVC. In both animals, expression of GFP was predominantly confined to a small region, possibly overlapping with the HVC shelf, a region located at the ventral border of HVC (Fig. 4.7). Of the around 20 animals we injected (using the same stereotaxic coordinates), only these two had an expression confined to the shelf, and it would therefore be interesting to test whether this is due a specific tropism of the vector.

Serotype 2/7

rAAV2/7 has been shown to transduce neurons in the mouse brain (e.g. substantia nigra and striatum) as efficiently as rAAV2/5, and much more efficiently than rAAV2, with retrograde transduction in different areas (Taymans et al., 2007). In our case, we found rAAV2/7 to be indeed better than rAAV2, but worse than rAAV2/5, with only very limited transduction resulting in only few
Figure 4.4: Light micrographs of cells retrogradely labeled neurons in HVC after injection of rAAV2/5 in RA (animal g20r7). Field of view: 887 µm x 609 µm.

Figure 4.5: Light micrographs of cells anterogradely labeled axons in Area X after injection of rAAV2/5 in HVC (animal g20r7). Field of view: 443 µm x 334 µm.
Figure 4.6: Retrogradely cells in HVC after injection of rAAV2/5 in RA (animal b4r6). In this animal more cells were visible, but only weakly expressing GFP. Field of view: 887 µm x 669 µm.

Figure 4.7: Injection site of rAAV2/6.2 in HVC. Similar to a second animal injected, the expression seems to be mostly confined to a small region ventral of HVC (animal p14r6). Field of view: 2.2 mm x 1.67 mm.
cells labeled (Fig. 4.8).

![Image](image.png)

**Figure 4.8:** Injection site of rAAV2/7 in HVC, with only few cells transduced (animal p1r6). Field of view: 2.2 mm x 1.67 mm.

**Serotype 2/8**

Similar to rAAV2/7, rAAV2/8 has also been reported to mediate efficient GFP expression in several mouse brain areas (Taymans et al., 2007). In the case of zebra finch, we found good transduction levels at the injection site (Fig. 4.9) and labeled axons in projecting areas, e.g. in RA after injection in HVC (Fig. 4.10), but no retrogradely transduced cells.

**Serotype 2/9**

Injection of rAAV2/9 resulted in a widespread expression in the posterior part of the forebrain following HVC injection in both animals (Fig. 4.11). An explanation for this transduction pattern may be found by comparison with other animal systems. In the adult mouse, self-complementary rAAV2/9, unlike other viral vectors, has been shown to be able to cross the blood-brain barrier, allowing to transduce motoneurons in the spinal cord after intravenous injection of the vector (Duque et al., 2009). Moreover, it has been shown that also single-stranded rAAV2/9 can cross the blood brain barrier, because intravenous administration of rAAV2/9 also leads in mice to an extensive and widespread gene delivery to various parts of the CNS (Rahim et al., 2011). It is therefore possible that, in our case, pressure injection of rAAV2/9 results in leakage of the fluid in the vascular system or more probably in the ventricle above HVC, and the vector can re-enter the brain at different positions. Due to the widespread expression
Figure 4.9: Injection site of rAAV2/8 in HVC, with good level of transduction (animal k2r6). Field of view: 2.2 mm x 1.67 mm.

Figure 4.10: Light micrograph of anterogradely labeled neurons in RA after injection of rAAV2/8 in HVC (animal k2r6). Field of view: 443 µm x 334 µm.
of the vector, we found difficult to determine if retrograde transduction took place, because we could not distinguish if spatially isolated cells were labeled because of retrograde transduction or because of transport of the viral particle through the vascular system. This vector may be of interest if a widespread delivery of a gene is necessary.

Figure 4.11: Widespread labeling in the posterior forebrain after injection of rAAV2/9 in HVC (animal k12r6). Field of view: 7.1 mm x 5.4 mm.

Serotype 2/rh10

AAVrh10 is a serotype evolutionarily similar to AAV8 which has been shown to transduce neurons in the pseudotyped form 2/rh10 (Cearley and Wolfe, 2006; Cearley et al., 2008). We found rAAV2/rh10 to only transduce few cells at the injection site, and we did not find any retrograde transduction.

Serotype 2/hu37

AAVhu37 also belongs to same evolutionary clade as AAVrh10 and AAV8 and pseudotyped with AAV2 has been shown to transduce neurons in different brain areas in mouse at intermediate levels (Cearley et al., 2008). Similar to AAV2/r10, we found only few cells expressing GFP at the injection site, and no retrograde transduction.

Serotype 2/rh8

AAVrh8 is a vector whose capsid does not share close homology to other known AAVs and which has been shown to transduce neurons at similar levels as
AAV2/hu37 in the AAV2/rh8 form (Cearley et al., 2008). In the case of zebra finch, we found more cells to be transduced at the injection site compared to AAV2/hu37, but also no retrograde transduction.

4.1.4 Results detail: self-complementary AAVs

The AAV genome, like all other paroviruses, consists of a linear single-stranded DNA molecule, which needs to be converted into double-stranded DNA before gene expression, in what is considered to be one of the major rate-limiting steps for transduction. The idea of self-complementary AAV (scAAV) is to circumvent this step by packaging both DNA strands as a single molecule, with the two halves of the single-stranded DNA molecule folding (most probably rapidly after uncoating) and forming a double stranded DNA of half the length (McCarty, 2008).

We tested 3 different commercially-available scAAV, all expressing GFP under the control of the CMV promoter.

**Serotype 2/1sc**

Self-complementary AAV2/1 did not improve transduction compared to the single-stranded version, with still no retrogradely labeled neurons.

**Serotype 2/5sc**

With the self-complementary version of rAAV2/5 we found some retrogradely labeled cells (Fig. 4.12), although less (but with intense expression) than with rAAV2/5ss. We used a long survival time, 7 weeks, and it could be interesting to test whether different survival time have an effect on the number of cells expressing the reporter gene.

**Serotype 2/9sc**

Similar to its single-stranded version, AAV2/9sc also resulted in spreading of the vector in the posterior part of the brain, although less widespread. We found cells labeled in HVC after injection in RA and we had the impression (but, due to the spreading, of course no proof) that they were in fact retrogradely transduced (Fig. 4.13).
Figure 4.12: Retrogradely labeled cell in HVC after injection of rAAV2/5sc in RA (animal p10r6). Field of view: 443 µm x 334 µm.

Figure 4.13: Low magnification image of the posterior forebrain after injection in RA of rAAV2/9sc. Similar to the single-stranded vector, a widespread expression can be observed. In HVC a cell is very strongly labeled, possibly by retrograde transduction (animal o5r6). Field of view: 7.1 mm x 5.4 mm.
Appendix A

Immunostaining

A.1 Introduction
In the initial part of my PhD project, I tested several antibodies against different neurotransmitters on wet (vibratome) sections. The motivation was to have a collection of antibodies ready to use for further studies, because only limited literature is available on immunohistochemistry in the zebra finch, while practicing and learning several procedures related to histological preparation. The antibodies tested were against neurotransmitter of enzymes including the inhibitory neurotransmitter $\alpha$-aminobutyric acid (GABA) and its synthesizing enzyme Glutamate Decarboxylase (GAD, the two different mammal isoforms GAD65 and GAD67 have been considered); the enzyme Tyrosine Hydroxylase (TH), which catalyzes the conversion of the amino acid tyrosine to dihydroxyphenylalanine (DOPA), a precursor of the catecholaminergic system; the enzyme Choline Acetyltransferase (ChAT), that catalyzes the joining of choline and acetyl CoA to acetylcholine; the inhibitory neurotransmitter Glycine; the proteins Synapsin I and Synaptophysin, which are both located at presynaptic terminals and involved in synaptic transmission.

A.2 Protocol
(Note: exact concentrations of antibodies, incubation times and blocking solutions are indicated in the following section.)
Zebra finch brain sections of 80 $\mu$m were cut with a vibratome and washed in three changes of PBS for 10 min each at RT. After washing, the sections were immersed in blocking solutions for up to 30 min at RT and next incubated with the primary antibody at 4°C overnight. This step was followed by three washes of 10 min each of PBS at RT. The sections were then reacted in Alexa Fluor
546 secondary antibodies for 1 hr at RT. Finally, sections were washed two
times with PBS and one with ddH$_2$O, mounted onto slides and coverslipped.
In some cases, immunolabeling was combined with tracer injections for local-
ization of some brain regions. Tracing was performed by injecting Alexa Fluor
488 dextrans (10000 molecular weight) in different brain regions.

A.3 Results

A.3.1 GABAergic system

**Anti-Glutamic acid decarboxylase 65/67 (Sigma G5163)**

Immunogen: synthetic peptide corresponding to the C-terminal region of hu-
man GAD 67.
Staining: Blood vessels.
Protocol: pre-blocking with 10% NGS 30 min, 5% during incubation with pri-
mary antibody (overnight 4°C).
Concentrations: 1:100, 1:200, 1:500, 1:1000.
Literature: -

**Monoclonal Anti-Glutamic acid decarboxylase 65 (Sigma G1166)**

Immunogen: purified rat brain GAD 65.
Staining: Terminals, but only in some regions (e.g. RA, Fig. A.1). Faint stain-
ing of cells overall.
Protocol: pre-blocking with 10% NGS 30 min, 5% during incubation with pri-
mary antibody (overnight 4°C).
Concentrations: 1:100, 1:200, 1:500.
Literature: -

**Monoclonal Anti-GABA antibody produced in mouse clone GB-69 (Sigma A0310)**

Immunogen: GABA conjugated to BSA.
Staining: Overall cells, however no structure shows exclusive expression of
GABA. Background too high. See Figs. A.2,A.3,A.4.
Protocol: no blocking; pre-blocking with 5% NGS 30 min and1% during incu-
bation with primary antibody (overnight 4°C).
Concentrations: 1:200, 1:400, 1:500, 1:1000.
Literature: Wonderschuetz and Bischof 2006 (personal communication with Bischof: they have the same problem with background).

**Anti-GABA antibody (Sigma A2052)**

Immunogen: GABA conjugated to BSA.
Staining: Overall cells, however no structure shows exclusive expression of GABA. In NIf some cells are stained, but apparently not the ones that project to HVC (see Fig. A.5). No staining in Uva (Fig. A.6). High background. Protocol: no blocking; pre-blocking with 5% NGS 30 min and 1% during incubation with primary antibody (overnight 4°C).
Concentrations: 1:200, 1:400, 1:500, 1:1000.
Literature: Fischer et al. 2007 (chicken).

**Rabbit anti-glutamate decarboxylase 65/67 polyclonal antibody (Chemicon AB1511)**

Immunogen: Synthetic peptide with a sequence from rat GAD65.
Staining: Terminals, cells weakly stained. Terminals and cells in RA (Fig. A.7), Area x (Figs A.8 and A.9), NIf (Fig. A.10, note that a dense population of terminals is present in NIf) and HVC (Fig. A.12). Terminals can be seen in Uva (Fig. A.11), but not cells. Protocol: no blocking; pre-blocking with 5% NGS 30 min and 1% during incubation with primary antibody (overnight 4°C).
Literature: Pakan et al. 2006 (pigeon).

**Rabbit polyclonal anti GABA (Chemicon AB131)**

Immunogen: GABA-gluteraldehyde-BSA.
Staining: Overall cells (Fig. A.13). For cells in RA see Fig.A.14.
Protocol: pre-blocking with 5% NGS 30 min and 1% during incubation with primary antibody (overnight 4°C).
Literature: -

**A.3.2 Catecholaminergic system**

**Rabbit anti-Tyrosine hydroxylase (Chemicon AB152)**

Immunogen: Denatured tyrosine hydroxylase from rat pheochromocytoma.
Staining: Terminals in several regions, e.g. HVC (Fig. A.15), IMAN (Fig. A.16), RA, NIf (Fig. A.17) and striatum (higher in Area X, absent in the
Globus Pallidus, Figs. A.19 and A.20). Some TH+ cells are visible in the midbrain (probably VTA and SNC). No terminals are visible in Uva.
Protocol: pre-blocking with 5% NGS 30 min and 1% during incubation with primary antibody (overnight 4°C).
Literature: Gale and Perkel 2006.

A.3.3 Cholinergic system

**Rabbit anti-ChAT 1465 (Donated by M. Epstein)**

Immunogen: chicken choline acetyltransferase.
Staining: Cells in the pallidum (Fig. A.21).
Protocol: pre-blocking with 10% NGS/1%BSA/Triton 45 min, same blocking solution during incubation with primary antibody (overnight 4°C).
Literature: Akutagawa and Konishi 2005 (note that axons in Uva are not labeled in our case).

**Rabbit anti-ChAT 2017 (Donated by M. Epstein)**

Immunogen: chicken choline acetyltransferase.
Staining: Cells in the pallidum (Figs. A.22 and A.23).
Protocol: pre-blocking with 10% NGS/1%BSA/Triton 45 min, same blocking solution during incubation with primary antibody (overnight 4°C).
Literature: Akutagawa and Konishi 2005 (note that axons in Uva are not labeled in our case).

A.3.4 Glycinergic system

**Rabbit anti-Glycine (Chemicon AB5030)**

Immunogen: Glycine-glutaraldehyde-BSA.
Staining: Very high background, no specific staining.
Protocol: pre-blocking with 10% NGS/1%BSA/Triton 30 min, same blocking solution during incubation with primary antibody (overnight 4°C).
Literature: -
A.3.5 Synapses

Anti-Synapsin I (Invitrogen A6442)

Immunogen: bovine Synapsin I.
Staining: No staining.
Protocol: pre-blocking with 5% NGS 30 min, same blocking solution during incubation with primary antibody (overnight 4°C).
Concentrations: 1:1000.
Literature: -

Anti-Synaptophysin I (Upstate MAB5258)

Immunogen: Vesicular fraction of bovine brain.
Staining: Synapses (fig. A.24).
Protocol: pre-blocking with 10% NGS 30 min, same blocking solution during incubation with primary antibody (overnight 4°C).
Literature: -

![Figure A.1](image1.jpg) **Figure A.1:** G1166 GABAergic terminals in RA. Scale bar 200 μm.

![Figure A.2](image2.jpg) **Figure A.2:** A0310 GABAergic cells in HVC. Scale bar 100 μm.
Figure A.3: A0310 GABAergic cells. Scale bar 50 µm.

Figure A.4: A0310 GABAergic cells. Scale bar 100 µm.

Figure A.5: A2052 GABAergic cells in the region of NIf (center of the image). Scale bar 100 µm.

Figure A.6: A2052 GABA immunoreactivity in the region of Uva: no cells are stained. Scale bar 50 µm.

Figure A.7: AB1511 GABA immunoreactivity in RA. Scale bar 100 µm.

Figure A.8: AB1511 GABA immunoreactivity in Area X. Scale bar 100 µm.
Figure A.9: AB1511 GABA immunoreactivity in Area X. Scale bar 50 µm.

Figure A.10: AB1511 GABA immunoreactivity in NIf. HVC. Scale bar 100 µm.

Figure A.11: AB1511 GABA immunoreactivity in Uva: terminals can be seen. Scale bar 100 µm.

Figure A.12: AB1511 GABA immunoreactivity in HVC. Scale bar 50 µm.

Figure A.13: AB131 GABA immunoreactivity. Scale bar 100 µm.

Figure A.14: AB131 GABA immunoreactivity in RA. Scale bar 50 µm.
Figure A.15: AB152 TH immunoreactivity in HVC. Scale bar 200 µm.

Figure A.16: AB152 TH positive terminals in lMAN. Scale bar 200 µm.

Figure A.17: AB152 TH positive terminals in NIf. Scale bar 100 µm.

Figure A.18: AB152 Overlay of TH immunostaining and NIf (green), retrogradely labeled by Alexa Fluor 488 injection in HVC. Scale bar 100 µm.

Figure A.19: AB152 TH positive terminals in NIf (green) and striatum. Scale bar 200 µm.

Figure A.20: AB152 TH positive terminals in anterior striatum. Note the sharp border. Scale bar 100 µm.
Figure A.21: ChAT 1465 Cholinergic cells in the pallidum. Scale bar 500 µm.

Figure A.22: ChAT 2017 Cholinergic cells in the pallidum. Scale bar 500 µm.

Figure A.23: ChAT 2017 Cholinergic cells in the pallidum. Scale bar 100 µm.

Figure A.24: MAB5258 Synaptophysin immunoreactivity (white) and retrogradely labeled cells (green). Scale bar 20 µm.


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