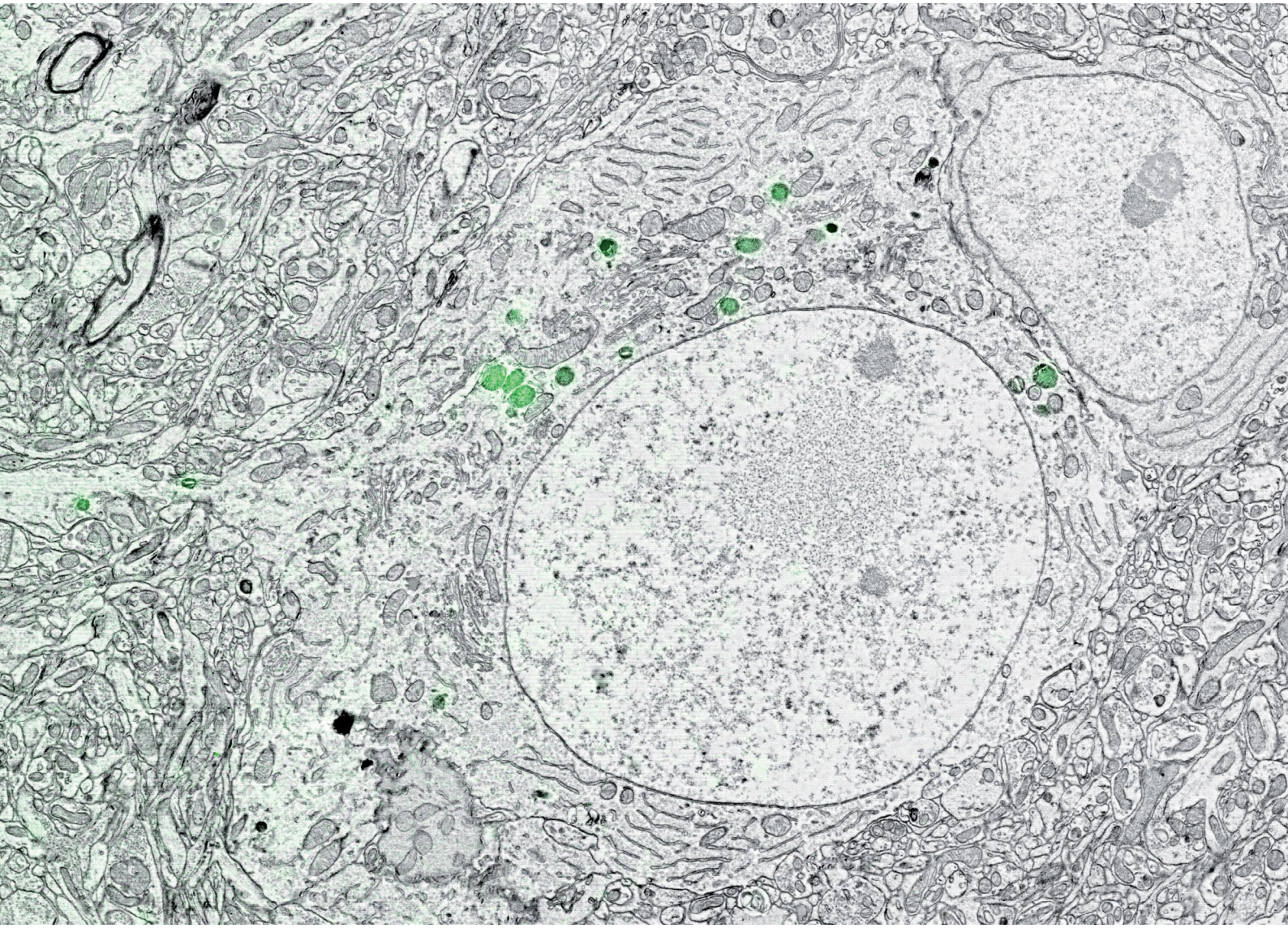
White Paper



Functional and Structural Investigation of Songbird Brain Projection Neurons with "Shuttle & Find"

Correlative Microscopy in Life Sciences

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Functional and Structural Investigation of Songbird Brain Projection Neurons with "Shuttle & Find"

Correlative Microscopy in Life Sciences

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Singing behavior of songbirds and human language share Many similarities. As language in humans, the song of the bird is not innate but learned from a tutor. Therefore songbirds are a good animal model to study brain areas and networks involved in this complex behavior. Similar brain structures in birds and human beings are assumed to steer those learning processes, as human language and birds singing are related processes. Therefore the understanding of learning processes in a songbird brain is a step towards understanding the learning and speech of language in humans.

Introduction

Within a songbird brain several regions are involved in song learning and production (Figure 1). Several brain areas are connected to the HVC region which is the main premotor area for vocal production. In this application connections within HVC are analyzed on an ultrastructural level. Cells projecting to the brain region Area X are labeled with a tracer and their connectivity within HVC can be investigated. Correlative Light and Electron Microscopy (CLEM) is essential for this application since both the identification of the projection cells in the HVC region via Fluorescence Light Microscopy (FLM) and ultrastructure of the same cells in Scanning Electron Microscopy (SEM) are necessary.

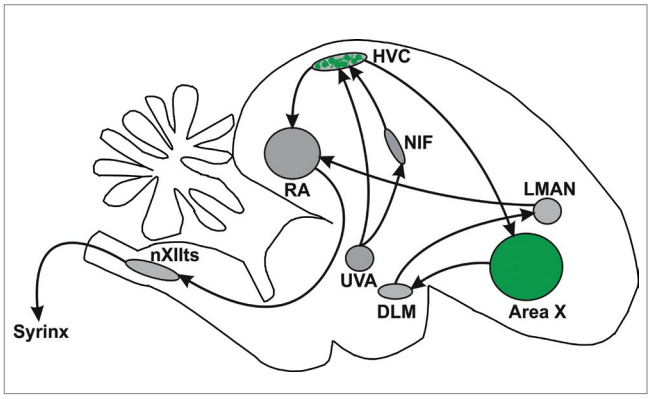

Figure 1 *Schematic representation of the songbird brain with regions involved in song learning and song production. Injection was made in Area X.*

Sample Preparation

The sample was prepared in a similar manner to the protocol described in detail in [1]. Area X of a zebra finch brain was located by stereotaxic coordinates and injected with 0.5 µl Alexa 488 dextran. After 5 days a perfusion with a solution of 2 % paraformaldehyde and 0.075 % glutaraldehyde in phosphate buffer (0.1 M, pH 7.4) was performed. Sagittal sections of the brain with 60 µm thickness were cut and washed in cacodylate buffer (0.1 M, pH 7.4), followed by a postfixation of 40 min in 1.5 % potassium ferrocyanide and 1% osmium tetroxide and then 40 min in 1 % osmium tetroxide, each in the same buffer. Finally the sections were postfixed in 1 % uranyl acetate in distilled water (40 min). After dehydration and embedding of the sections in Durcupan ACM resin, they were cured for 48 h at 52° C. The area around HVC was resected, attached to a blank resin block, and cut in ultrathin sections of a thickness in the range of 60-90 nm. The sections were transferred onto cover slips coated with ITO (Indium Tin Oxide, 8 Ω/sq). This coating reduces charging

in SEM and a high optical transparency is still assured. Finally they were stained for 2 min in Reynold's lead citrate and washed 3 times for 30 sec in ddH_20.

Imaging

The cover slip was placed into the sample holder especially designed for CLEM by ZEISS. This holder can be used in light microscopy as well as SEM so that the sample is stably fixed in the holder during the whole imaging process. The holder has three fiducial markers which define a coordinate system that can be calibrated very fast and semi-automatically in the Shuttle & Find module of the AxioVision Software.

FLM of the sections was performed with Axio Observer.Z1 (Carl Zeiss Microscopy GmbH) using a 100 x objective (EC Epiplan-Neofluar 100 x/0.90 HD DIC) and a filter set with 470/40 nm excitation and 525/50 nm emission (Filter set 38 HE). The microscope was equipped with an AxioCam HRm. Regions of interest (ROIs) were defined and selected in the fluorescence image.

Next the sample was transferred to a SUPRA® 40VP SEM (Carl Zeiss Microscopy GmbH). The holder was calibrated semi-automatically and after a few seconds the frame imaged in the FLM was located. A subsequent fine calibration allows to image the selected ROIs at a precision below 5 µm. SEM imaging was done at an acceleration voltage of 1.5 kV with the in-lens secondary electron detector.

Results

Figure 2 shows a widefield FLM image of an ultrathin section from the HVC area. Fluorescent spots indicate cell compartments where the tracer is localized. Thus, a neuron projecting from HVC to Area X is identified and selected as ROI.

The selected ROI is further shown in Figure 3. Figure 3a is an enlarged section of Figure 2. Figure 3b shows the structure of the selected neuron imaged in the SEM. The overlay of FLM and SEM images of this ROI is displayed in Figure 4 and verifies that both microscope systems really image the same ROI. The highest fluorescence is located in specific vesicular compartments where the tracers, which distribute through the entire cell, have the highest concentration.

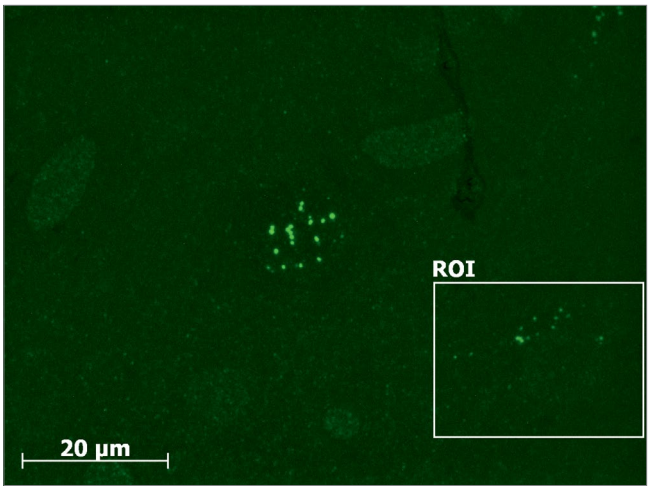

Figure 2 *FLM image of an ultrathin section with highlighted ROI.*

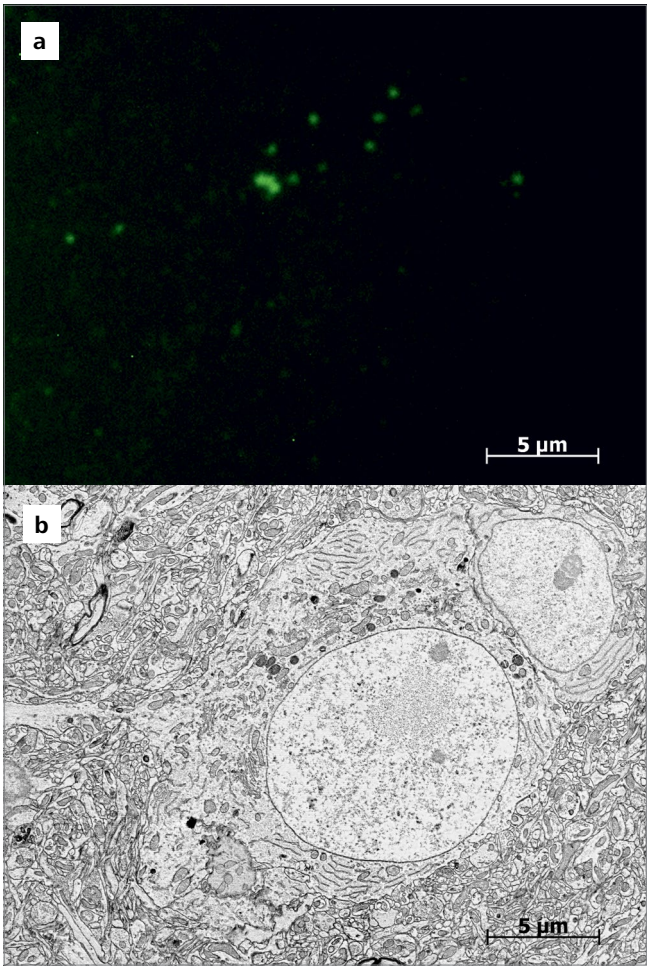

Figure 3 *FLM (3a) and SEM (3b) images at ROI selected in Figure 2.*

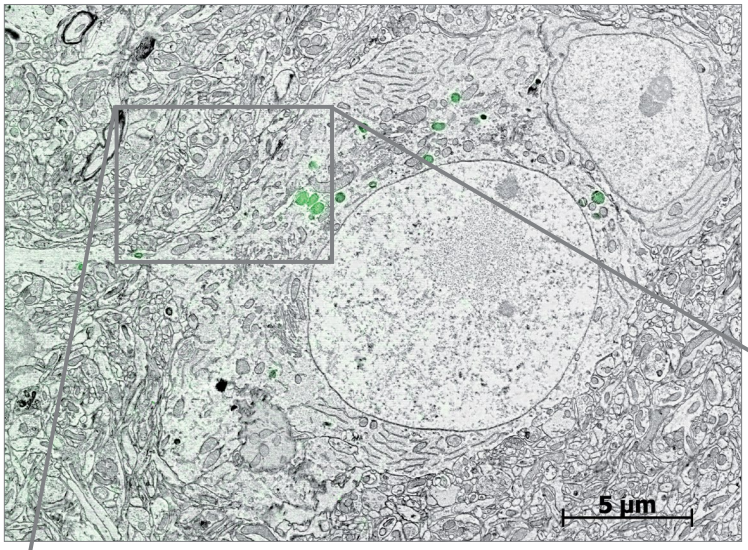

Figure 4 *Overlay at ROI selected in Figure 2.*

Figure 5 shows the upper left part of the selected neuron at higher magnification in an overlay of FLM and SEM. The SEM image has a pixel size of 4.6 nm which is sufficient for this application as it clearly resolves the ultrastructure of subcellular structures in the neuron as mitochondria (A), myelinated axons (B) or synaptic vesicles (C).

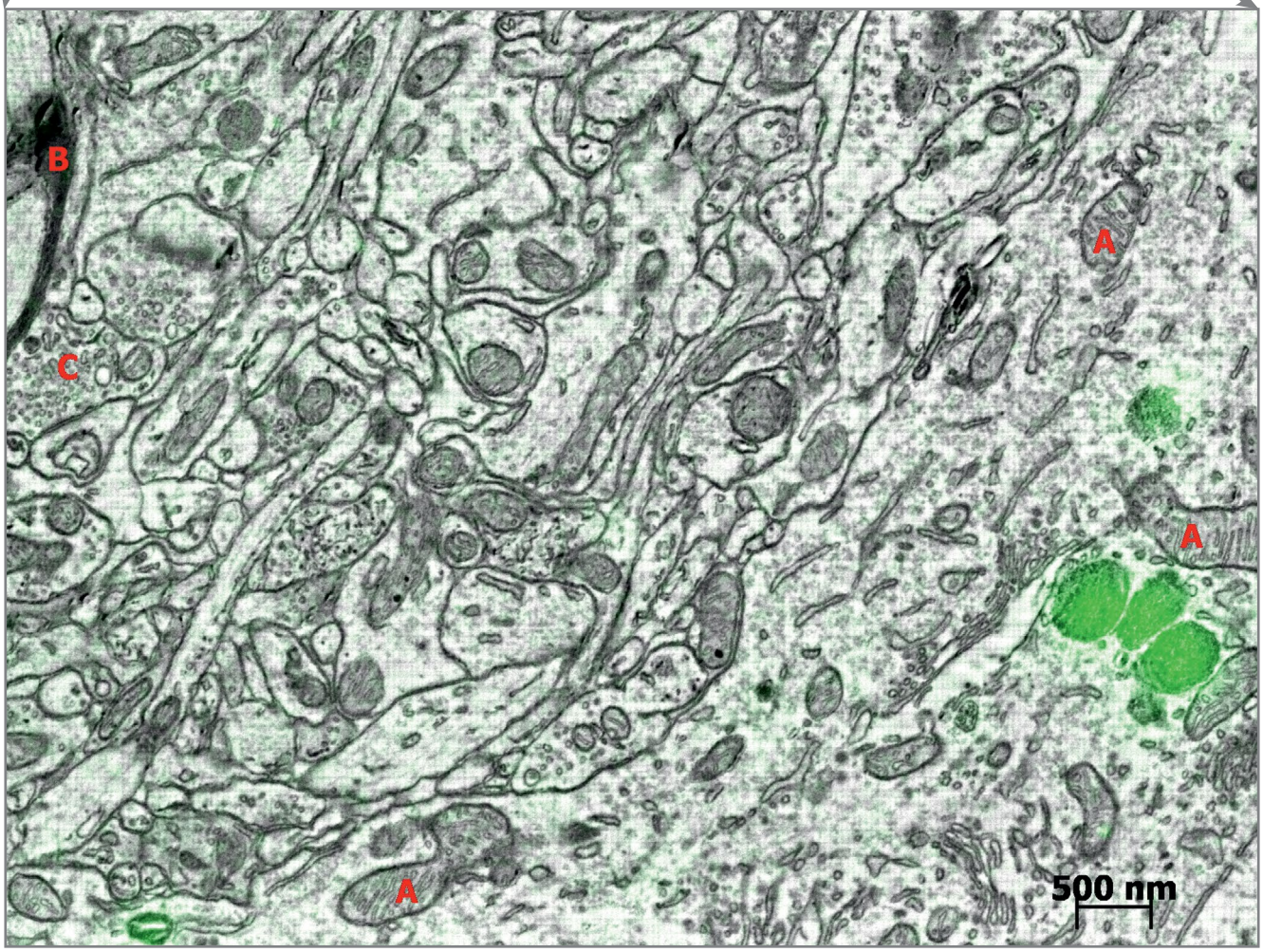

Figure 5 *Higher magnification overlay within Figure 4; SEM image clearly shows the structure of mitochondria (A), myelinated axons (B) or synaptic vesicles (C).*

Conclusion and Outlook

The Shuttle & Find interface for Correlative Microscopy enables fast and reliable high-resolution context imaging of specifically connected neurons. They are labeled by fluorescent tracers and imaged in FLM as well as in SEM. The overlay of both information allows for classification of neurons observed in the SEM based on information from LM. At the same time SEM imaging of the same sections gives ultrastructural information, e.g. presence of active synapses, and makes segmentation possible.

With the Shuttle & Find solution searching of the same position in both microscopes is automated, the workflow is therefore sped up and serial section imaging can be performed at a moderate time. Although this application example shows only one fluorescent color, it is technically feasible to extend the technique to multicolor tracing and imaging. By using different colors for different neuron types, the network can be characterized.

References

[1] D. Oberti, M.A. Kirschmann and R.H. Hahnloser, Front. Neuroanat. 4:24 (2010) doi: 10.3389/fnana.2010.00024

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