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Acceleration of Neuroscience Research Discovery by Incorporation of Large Area/Volume Microscopic Data Workflows

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Modern microscopes are becoming increasingly complex instruments enabling registration of image sets far beyond a single field of view. This is being achieved by integration of sophisticated scanning stages, capable of moving the field of view in precise synchrony with acquisition, providing reliable meta data encoding time, space and multiple imaging modalities. As a result, increasingly complex multi-dimensional microscopic data sets are being generated and analyzed. In addition to executing different imaging modalities, the modern instruments are capable of sample manipulation, which further increases the complexity and size of the data sets that are routinely being analyzed. For all these reasons, multifaceted workflows are required from sample preparation, through imaging to structuring and analyses of the image data. Normal and pathological studies of neuronal networks are a prime example of where complex image data facilitates our understanding of structural organization of neuronal circuits and the specific domains of emerging pathological conditions. The needs of large image data sets is amplified by the fact that in the central nervous system (CNS), cell bodies, axonal terminals and dendritic processes span very large volumes, which ideally should be included in the image sets at appropriate resolution. On the other hand, it is important to identify the pathological process at its emergence, where only rare events, representing foci of the nascent pathological process are present in otherwise normal brain tissue. In this talk, we provide several examples of how modern light, electron and correlative microscopy facilitated our effort to identify the underlying pathology associated with feeding and swallowing deficits present in a mouse model of DiGeorge Syndrome (22q11DS, LgDel).

Tissue clearing techniques enabling the imaging of whole organisms have exploded recently and dramatically enhanced our perception of brain architecture and connectivity. Our group uses a 100-year-old technique - BABB [3], [2], to clear whole mouse embryos with the goal of identifying abnormal axonal growth of trigeminal motor and sensory neurons in LgDel mouse. We combine tracing with biocytin/Alexa Fluor and wide dynamic range confocal imaging to produce image sets that support reliable reconstruction of a single neuron and its processes, spanning from the rhombomeres to anterior tip of the mandibular branchial arch. We use hybrid detector – a GaAsP photocathode coupled to avalanche photomultiplier in photon counting regime on Leica SP8. Thus, our experimental setup allows us to register on the same large volume of the embryo, the signal from areas with high photon yield, such as the injection site to very low photon readouts, such as the smallest neuronal processes. Sampling rate was kept at 10% of the excitation rate (80MGHz) of the source to achieve linearity. An oil immersion objective lens matches the refractive index (RI) of BABB and offers large working distance to capture half of the embryo (500 μm). As a result of matching RI, the spherical aberrations are minimized leading to the production of robust 3D volumes and segmentation of the axonal trajectories. Unlike BABA, newer clearing approaches such as Clarity do preserve fluorescence from reporter proteins. Additionally, we have also applied passive Clarity clearing, which requires a highly specialized lens (Leica HC Fluotar L 25x/1.0, IMM (n=1.457)), but produces image sets with unparalleled quality.

Whereas the light microscopy imaging technology had improved dramatically, the resolution is still limited close to diffraction. Moreover, the amount of cellular and tissue information is restricted to a

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relatively few labeling colors that can span the visible spectrum without interference. Electron microscopy (EM), in principle, surmounts these deficits, since with simple heavy metal counterstain such as uranyl acetate one can explore the full spectrum of cellular and tissue morphology. Until recently the majority of subcellular structure analyses required transmission electron microscopy (TEM) imaging from ultrathin sections, which imposed a size limitation. The architecture of the scanning electron microscope (SEM) allows for accommodation of larger samples, but the signal is relatively weak at the detector. New improvements in SEM including, sample logistics [1], stable and monochromatic field emission electron sources, solid state detectors, and the ability to section the sample with either the destructive focused ion beam (FIB) or an ultramicrotome in the chamber has broadened the spectrum of biomedical applications performed in the SEM. Many SEM instruments can achieve block-face imaging using elastically backscattered electrons, providing images similar to the TEM. We study the hypoglossal motoneurons in LgDel mice with FEI Helios 600 FIB SEM equipped with concentric ring backscatter detector. To minimize the charging artifacts, we cut a large ultrathin section (120 nm, 5x3 mm) of the entire brainstem, which are placed on semiconductor grade Si-wafer. Using FEI MAPS software, the image acquisition is managed in layers. First layer represents stitched tiles of the entire sample at low magnification (600x), which is used as navigation map. This layer allows us to precisely focus on hypoglossal nucleus based on its characteristic cytoarchitectural features. The next layer represents high magnification and high resolution (80,000x, 1.6 nm/pixel) tiles of adjacent fields of view stitched together. High-resolution image sets are linked to the low-resolution map allowing for precise localization. Large area, high-resolution SEM image sets can be as large as 400x350 μm with a pixel size of 1.6 nm. This approach enabled us to identify with confidence sparse, but consistent neuronal degeneration in LgDel mice, in addition to distinct cellular abnormalities including Golgi-stress, decreased number of organelles and mitochondria structural abnormalities. In addition, 3D image sets generated using FIB SEM and VolumeScope, enabled us to define, in detail, the degree of cellular abnormalities associated with the LgDel.

The above SEM approach provided excellent structural detail analogous to TEM, however osmium fixation compromises antigenicity, making these samples unacceptable for post embedding immuno-gold detection of proteins. To surmount this inherent deficit, we took two approaches. We devised a correlative light electron microscopy (CLEM) workflow based on integrated low/high resolution layered data sets. Low-resolution image sets include the entire coverslip, which was later in the pipeline aligned to the low-resolution SEM counterpart. High-resolution tile scans were also stitched and overlaid for cellular and subcellular identification. Such data integration dramatically facilitated the identification of the same field in LM and EM. We further refined existing post-embedding approaches either using osmium fixed material (for amino acids detection) or high pressure frozen for protein detection (Leica EM ICE) followed by freeze substitution and low temperature embedding system (Leica EM AFS2) to make the sample suitable for imaging large area using SEM. These approaches provided good structural preservation with reliable antigen detection with 10 nm gold particles [4].

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