論 文 の 内 容 の 要 旨

論文題目　Three-dimensional image analysis of high resolution confocal microscopy data of the Drosophila melanogaster brain

（キイロショウジョウバエ脳の高解像度共焦点顕微鏡データの三次元画像解析）

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The brain has for a long time attracted the fascination of scientists and philosophers. However, as the main organ of our nervous system and due to its extreme complexity, the brain investigation is, even nowadays, a challenge for all neuroscientists. Direct analysis of the human brain is hampered from complex technical and ethical problems, and scientists quickly opted for indirect research using animal models. Among them, the fruit fly Drosophila melanogaster has been one of the most popular to investigate a variety of fields from genomics to neurosciences. Many tools have been developed to manipulate the fruit fly genome, offering opportunities to investigate other fields such as developmental and functional neurosciences leading to many discoveries, which, under certain conditions, can be extrapolated to human research. Those tools include the insertion of transgenic genes to drive or inactivate the expression of proteins in specific cells of the fruit fly. In particular, this technology enables the expression of fluorescent proteins in the cytoplasm or membrane of certain neurons. This fluorescence can be captured by a confocal microscope to reconstruct three-dimensional images of the labeled neurons.

The brain of an adult fruit fly is a complex network of about 100,000 neurons projecting in a 600-µm wide, 300-µm tall, and 160-µm thick volume. Its cell bodies are mainly located at its surface (outer part) whereas the neurites innervate the inner part (synaptic neuropil). The fly brain features a wide variety of neurons that can be categorized by the types of neurotransmitters released in synapses. Among all those categories, we will focus on two specific neuron types: the octopaminergic and dopaminergic neurons and their connections. Octopamine, is an endogenous trace amine exclusively found in invertebrate organisms. In insects, this molecule mediates a wide range of functions. A Drosophila melanogaster brain counts around 100 octopaminergic neurons and their organization is well conserved among invertebrate organisms proving their importance. On the other hand, dopamine is a neurotransmitter and neurohormone found in both vertebrates and invertebrates. This molecule plays major roles in the human brain and body, and dysfunctions of the dopamine system can lead to serious diseases such as Parkinson’s disease or schizophrenia. Octopaminergic and dopaminergic neurons appear to be determinant in the correct operating of the central nervous system and a precise and systematic analysis of their evolution and interaction is necessary to understand its mechanisms.

Interestingly, unlike most other neurons, those two types monoamine neurons feature very complex structures with many thin branches that are densely spread in the whole brain, multiplying the connections with other neurons. Because of this complexity, three-dimensional images of very high resolution (0.2μm square voxels) are necessary to characterize their morphology. Confocal microscopy is a powerful tool that can be used to obtain a stack of serial section images of the signal distribution by illuminating the sample point-by-point within its volume with a laser beam. The fluorescence emitted from each illumination position is recorded as the signal level of each voxel. However, even though the specimens are made sufficiently transparent, they are never completely clear and optically uniform. Thus, the excitation light and the light emitted from the fluorescent objects are attenuated, refracted and scattered by the sample tissue, especially in thick samples. The light coming from deeper parts of the sample is partially affected and the signal is blurred by random refraction and scattering of the light ray, significantly reducing the image quality in those regions because less photons will reach the photodetectors causing a diminution of sensitivity and increase of the noise to signal ratio. Moreover, as the laser beam goes through the sample, the neighboring regions of the focus point are slightly photobleached leading to a diminution of the signal intensity and a loss of contrast. In conclusion the image quality in laser scanning microscopy techniques decreases with the depth from the sample surface and deeper objects appear blurry, sometimes fused with their neighbors if the focal plane is deeper than 100 μm.

Octopaminergic and dopaminergic neuron branches spread in the whole 160μm-thick fly brain, deeper than the 100-μm theoretical limit over which the confocal scanning microscopy image will not be clear enough for high-resolution study. Several workaround techniques have been employed to overcome this problem. Because our specimen is thinner than 200 μm along z-axis, we can record the images from both sides of the samples: from the front toward the center and from the other side. For taking such set of images, the sample is mounted between two thin cover slips, and the image stacks are captured from both sides by flipping the sample after the first scan. Both image volumes must be a little bit thicker than half of the brain to ensure overlap of the two stacks. Using these overlapping sections from both images as a guide, they can be concatenated, or stitched. Although various programs have been developed for stitching such volume data, they compensate only translation and rotation around the optical axes (z). Straightforward concatenation is often not possible, because the small rotations that occur when the sample is flipped involves not only the rotation around the z axis but also around x and y (tilting). Before stitching the front and back stacks, they must be registered in such a way that each neuronal fiber appears contiguous at the boundary of the two stacks, because a gap in the final image will affect neuron tracing and further study.

To address this problem we implemented an algorithm to compute the best relative position of each stack and compensate the tilting around x- and y-axes, and developed a plug-in module for the industry-standard platform for three-dimensional image manipulation called FIJI/ImageJ. Our technique involved the computation of local features called SIFT (scale invariant feature transform) to achieve a robust registration of big image stacks. The SIFT technique, introduced by D. Lowe in 1999 is a computer vision algorithm detecting and

describing local feature vectors in images. The set of feature vectors of two similar images will also be similar, and matching those features is equivalent to an image comparison. The location of those similar features can be used as landmarks to compute a transformation model (translation and rotation) between the two images. The SIFT algorithm can be summarized in three steps; feature extraction when the locations of the feature vectors are extracted, feature matching when the comparable vectors between the two images are identified, and transformation model computation when the coordinates of the features of one image stack is aligned to those of the other stack.

The algorithm we developed based on this technique is a four-step procedure that can be iterated until satisfying results. First, the common part of the neighboring (front and back) stacks is extracted (a), then the rotation and tilt are fixed progressively around z-axis (b), x-axis (c) and y-axis (d) by comparing cross-sections of similar positions within the overlap along the three cross-section planes.

The overlap extraction can easily be done under a simple assumption: the transformation, especially tilting induced by the image acquisition is small enough to keep a good similarity between image slices. The last slice of the front stack is then compared to all the sections of the back stack. The slice showing the strongest similarity (highest number of matching SIFT feature vectors) is selected as the boundary of the overlap. Within this common part, the cross-sections of the volume data at the same depth are compared and a first transformation model of rotation around the optical axis (z-axis) and translation along y-axis and z-axis is computed and applied to the back stack (b). Similarly, the cross-sections parallel to the (y, z) plan of the roughly registered back stack, are compared with the one of the front stack located at the same position (c). A new transformation model of rotation around x and translation along the other axes is computed and concatenated to the first one. Finally, the rotation around y and the remaining translation are identified (d) by comparing the cross-sections parallel to (x, z). This way, the back stack is progressively matched to the front stack, and several iterations can improve the continuity of the final alignment.

However, because the limitation of imaging depth in confocal microscopy and the photobleaching that occur during image stack acquisition, the intensity and image quality of the corresponding optical sections in two overlapping stacks often appear somewhat different. Even though SIFT is in principle robust against inconsistent luminosity in the two images, in practice standardizing, even roughly, the brightness and contrast between the two stacks often gives better registration results. We therefore developed another software module that compensates brightness and contrast of serial section images within the stacks. Because the deeper slices usually appear darker than the upper ones it is often necessary to increase the intensity of the signal of each section of the image stack non-uniformly. For doing this we set two intensity correction values, one for the first slice and the other for the last, and the intermediate slices are enhanced using interpolated parameters between those two values using one of the three transition curves, following a constant, linear or exponential law.

Precision of our SIFT-based image registration algorithm should be validated for finding the best parameters required for registration. However, because we can never know the precise amount of rotation in real samples, we have to make a mock pair of image stacks one of which is made from the same stack with the other but is rotated arbitrarily with known angles. Simple extraction and rotation of an image substack is not suitable for this purpose, because image clarity and noise level depending on the distance from the cover slip should also be simulated. To this purpose we created a simulator that generates artificial images that mimic laser scanning image stacks. It is known that confocal microscopes induce a noise in the data that follows a Gamma law of shape k and scale θ. We computed the empirical parameters along z-axis of the noise of our microscope by fitting a Gamma low to five real images, in the signal (fibers) and in the background, separately for the front and back stacks. A binary stack of tubular structures of neurites, generated by applying a threshold filter to a real image stack, was then cut into two overlapping substacks, and average values along z of those parameters were used as noise filter to reproduce realistic confocal three-dimensional images of fibers. The back stack was rotated and tilted at pre-defined angles before filtering. The resulting pair of image datasets were submitted to our registration algorithm and we found that the front and back substacks were correctly aligned after only two iterations of SIFT comparison.

Using this technique, we can now generate image stacks of the entire fly brain with very high optical resolution, which is suitable for analyzing fine and complex neural fiber branches such as those of the octopaminergic and dopaminergic neurons. We aimed at comparing the projections in the brains under different circumstances and performed two experiments. The first one, referred to as “age-dependency analysis” analyzes the morphological evolution of the octopaminergic and dopaminergic neurons over time by qualitatively and quantitatively comparing their arborization and connectivity patterns in the brains of young flies (5 day-old) and old flies (30 day-old). The second one, the “activity-dependency analysis”, examines the effect of activity-dependent structural plasticity in the neural networks of monoamine cells. To this purpose we inactivate octopaminergic or dopaminergic neurons by cell-type specific expression of the inward-rectifier potassium ion channels called Kir2.1, which has the particularity to be opened at a resting membrane potential. This causes a massive flux of potassium through the membrane inducing its hyperpolarization. We have taken seven datasets of octopaminergic and dopaminergic neurons and their pre-synaptic sites of young and old flies as well as old files that are raised with and without cell-type specific neuron inactivation.

We could show differences between those neurons under various condition. Octopaminergic neurons seem to be less subject to the effect of time or inactivation than dopaminergic neurons that degenerate quickly after inactivation but also, at a slower pace, within time. However, dopaminergic neurons are crucial for Drosophila melanogaster, who cannot survive more than a few days after inactivation. This suggest that the function of these neurons cannot be ensure by any other type of neurons.