Digital Reconstructions of *Drosophila* FraX Neurons from Image Stacks

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INTRODUCTION

Fragile X Syndrome (FraX) is the most common inherited mental retardation disease. The disease results from the silencing of the *fragile X mental retardation 1* (*fmr1*) gene which encodes for the fragile X mental retardation protein (FMRP). FMRP is a widely expressed translational suppressor with many potential regulative targets.

In the *Drosophila* model of FraX, dFMRP has been shown to be a potent translational suppressor of neuronal complexity and synaptic differentiation. Subsequently, *Drosophila fmr1* null mutant neurons have increased dendritic elaboration and axonal branching (Fig. 1). Overexpression of dFMRP, on the other hand, results in reduced neuronal complexity.

An effective way to analyze the morphology of *Drosophila* FraX neurons is via digital reconstructions from image stacks. Digital reconstructions allow for analysis of 3D neuronal structure, and provide morphometric data such as branch number, branch order, average diameter, total path length, and total surface area.

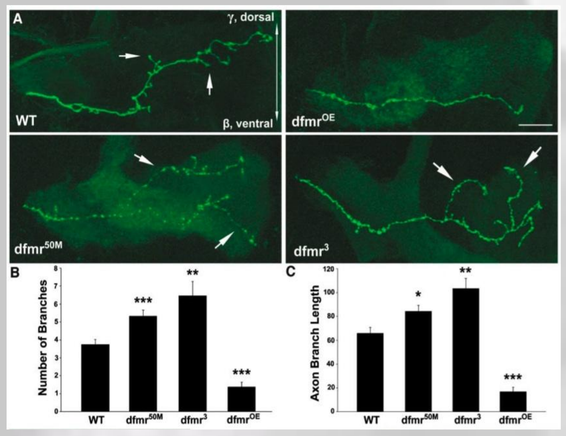

Figure 1. Data from collaborators that indicates dFMRP negatively regulates axonal branching in *Drosophila* mushroom body Kenyan cells (5). WT=wild-type; dfmr^{OE}=dFMRP overexpression; dfmr^{50M} and dfmr^{3}=two types of *dfmr* deletion mutants. A) Comparison of single WT and mutant Kenyan cells labeled via the MARCM technique. Arrows indicate small branches of interest. B)/C) Branch number and axonal length comparisons. *=significance 0.001<p<0.05; **=0.0001<p<0.001; ***=p<0.0001.

Why use the *Drosophila* Model of FraX?

•dFMRP has similar structure, RNA-binding properties, subcellular expression patterns, and functional roles as mammalian FMRP.

•*dfmr1* mutants show more prominent phenotypes than mice *fmr1* knockouts.

•Single, *Drosophila* mutant neurons can be identified *in situ* for analysis of neuronal structure.

•One drawback of the *Drosophila* FraX model is that it is difficult to perform learning and memory assays.

METHODS

The raw data was received from our collaborators in the form of confocal image stacks of *Drosophila* FraX neurons. Single Kenyan cells (Fig. 2) from the *Drosophila* mushroom bodies (Fig. 3) were labeled *in situ* using the MARCM technique.

Figure 3. Model of the Drosophila brain (6). Red and yellow=visual processing areas; blue=antenna processing areas; brown=mushroom bodies. Mushroom bodies are involved in olfactory learning and memory.

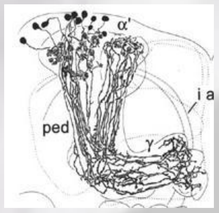

Figure 2. Diagram of Kenyan cells in the *Drosophila* mushroom body (3).

MARCM Technique

- Utilizes a repressible cell marker that exclusively labels single mutant neurons *in situ*.
- Used with genetically mosaic organisms--organisms that have specific tissues/cells with different genotypes but are otherwise wild-type.
- Previous techniques negatively label mutant neurons and thus do not show the neuronal structure.
- How it works (Fig. 4):
  1. Develop strains of genetically mosaic organisms in which a cell marker repressor gene is placed in *trans* (on the opposite homologous chromosome) to a mutant gene of interest.
  2. FRT sites upstream of the genes of interest cause mitotic recombination between homologous chromosomes, resulting in some of the daughter cells being homozygous mutants.
  3. Homozygous mutant cells are exclusively labeled due to loss of the repressor.

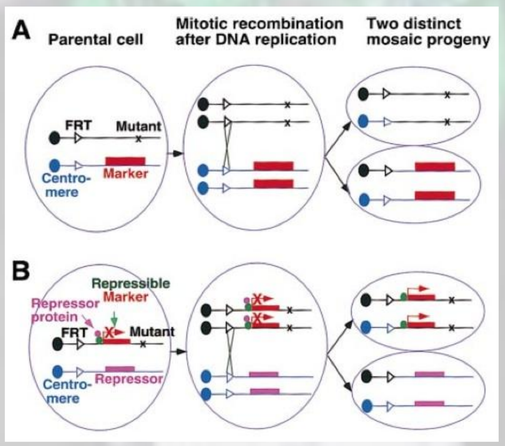

Figure 4. Diagram of two techniques used to label mutant clones in mosaic organisms (4). A) The traditional method in which homozygous mutant progeny are negatively labeled. B) The MARCM technique--homozygous mutant progeny are exclusively labeled due to loss of the repressor.

GAL4-UAS Repressible Expression System

•Our collaborators used the GAL4-UAS expression system for the MARCM technique (Fig. 5).

•GAL4 is a transcriptional activator from yeast that binds to the UAS (upstream activation sequence). Target genes can be inserted downstream of the UAS.

•*CD8-GFP* gene was used as a cell marker (CD8---> immunohistochemisty; GFP---> fluorescence)

•*GAL80* gene was used as a repressor of the cell marker. GAL80 functions as a repressor by binding to the UAS sequence, thereby inhibiting GAL4.

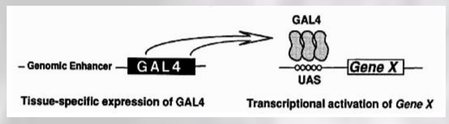

Figure 5. Diagram of the GAL4-UAS expression system (1).

Computer Applications

Two programs were used to create the digital reconstructions from image stacks: ImageJ/Neuron_Morpho and MIEN. ImageJ is a public domain Java image-processing program. Neuron_Morpho is a plugin for ImageJ that collects neuron tracing data.

MIEN (model interaction environment for neuroscience) is an application developed to analyze neuroanatomical and time series data. This application was used to 1) View the 3D reconstructions, and 2) Calculate the morphometric data.

Tracing

The confocal neuronal image stacks were semi-manually traced using ImageJ/Neuron_Morpho (Fig. 6-8). The data recorded from tracing is concise and includes:

•X, Y, Z coordinates of neuron

•Diameter of neuron

•Part of neuron (soma, dendrite, axon, bifurcation, end point)

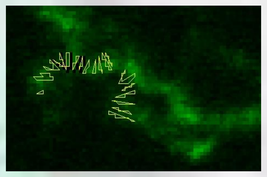

Figure 6. Example of neuronal tracing process. Image stacks are highly zoomed during tracing to acquire precise diameters.

Figure 7. Still image of tracings. Z projection of neuron is shown below.

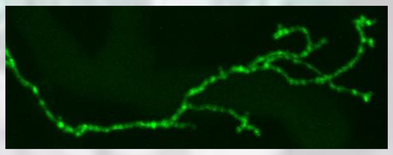

Figure 8. Z projection (slice integration) of image stack.

RESULTS

Digital Reconstructions of Neurons

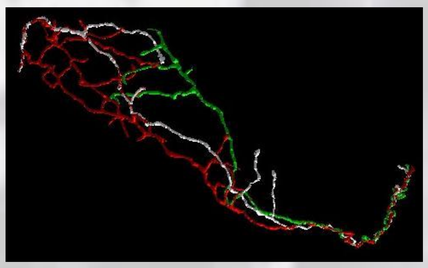

Figure 9. 3D neuronal reconstruction of intertwined axons. Z projection of neuronal image stack is shown below.

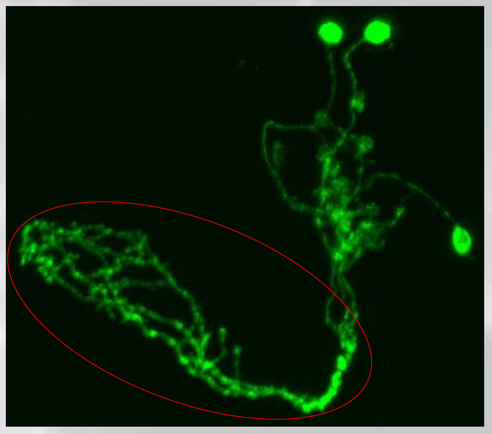

Figure 10. Z projection (slice integration) of image stack. The red circle indicates the part of the neurons that has been digitized.

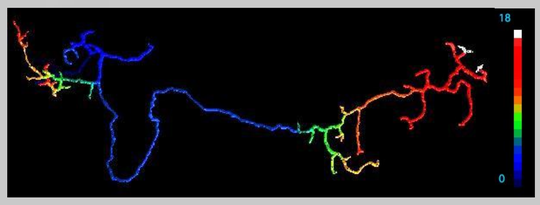

Figure 11. Neuronal reconstruction showing branch order gradient. Dark blue/violet=lowest branch order; white=highest branch order. The maximum branch order of this neuronal reconstruction is 18.

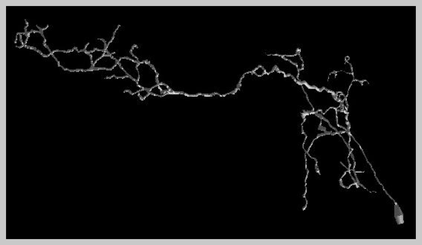

Figure 12. 3D reconstruction of a whole neuron. Z projection of neuronal image stack is shown below.

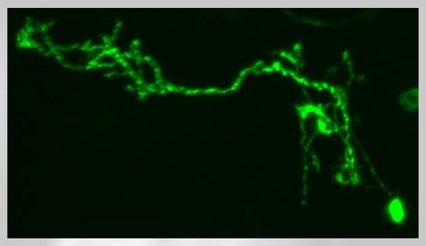

Figure 13. Z projection of image stack.

Morphometrics

Total path length, total surface area, maximum branch order, and number of bifurcations are highly variable between neuronal reconstructions (Table 1; Fig. 14). Average diameter was the only parameter that remained relatively constant between the neuronal reconstructions (Fig. 15).

Morphometric Data								
	Neuron 1	Neuron 2	Neuron 3	Neuron 4	Neuron 5	Neuron 6	Neuron 7	Neuron 8
Number of bifurcations	16	14	9	14	22	39	23	11
Total path length (um)	679.821	443.199	581.982	366.450	474.027	909.184	1078.318	568.790
Average diameter (um)	0.556	0.484	0.676	0.620	0.677	0.618	0.544	0.526
Total surface area (um^2)	1261.840	708.644	1273.557	736.231	1028.239	1749.758	1978.611	968.165
Maximum branch order	13	12	7	12	16	18	11	11

Table 1. Morphometric data from eight neuronal reconstructions.

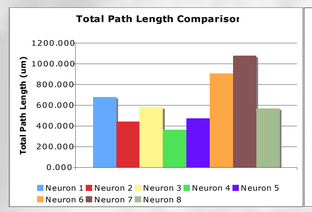

Figure 14. Total path length results from eight neuronal reconstructions.

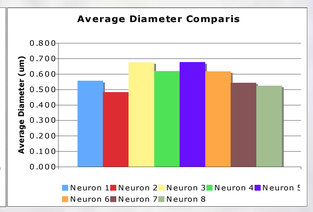

Figure 15. Average diameter results from eight neuronal reconstructions.

FUTURE WORK

•Obtain, digitize and compare image stacks of wild-type neurons.

•Overlay reconstructions with respect to the mushroom body.

•Create an online database of the digital reconstructions and morphometric data.

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