

# The high density of mitochondria in a region of axonal overlap can suggest the presence of en passant synapses.

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## Introduction

As the primary energy supplier at the cellular level, mitochondria must be present in the regions of a cell where the demand for their product is greatest. Because of the need for mitochondria to be in abundance in regions with high energy needs, their presence is typically a marker for high-energy activities. In neuronal cells, these activities include endocytosis and exocytosis. As a result, neuronal cells would be expected to have high concentrations of mitochondria in the cell bodies, where all the creation machinery is located, in growth cones, where high amounts of sol/gel actin transformations occur, each requiring energy, and at chemically synaptic nerve terminals. At a chemical synapse, a great deal of endocytosis and exocytosis occur, and therefore many mitochondria must be present in order to accommodate the energy demands of the cellular region. Using these observations, it was decided that there was a correlation between mitochondrial density and regions of high energy need in neuronal cells.

With this information in hand, it was decided to look for the presence of en passant synapses, chemical synapses formed by crossing axons, by looking for regions of overlapping axons, and their mitochondrial densities. It is postulated that in some regions of axonal overlap, high densities of mitochondria should be present, indicating that a chemical synapse is possible at that location. Another possibility is the formation of an electrical synapse, the result of a series of gap junctions forming between two neurons. This type of synapse is passive, and requires that the charged ions travel through the pores, essentially connecting the cytoplasms of the two axons.

The procedure for detecting mitochondria in the neuronal cells involves the use of the fluorescent dye Rhodamine-123. Rhodamine-123 is a mitochondria-specific dye; it only labels mitochondria specifically, and no other cellular structures (Johnson, 1980). When excited by a 485 nm light, the dye emits a green photon, causing the molecules, and whatever structure they are attached to, to fluoresce green. This dye is an ideal dye for the intended applications due to the high-contrast images it facilitates, and because it is both non-cytotoxic and a vital dye (Johnson, 1980). Since the use of this dye does not require the fixation of the target cells, the cells can be observed in conditions closer to their native environment. The dye also provides for a simple method of quantifying the mitochondrial density: luminosity. As the mitochondria become less dense, so does the concentration of rhodamine-123 molecules over a certain area. Therefore, the higher the density of mitochondria is in a region, the higher the density of rhodamine-123 will be in that region. This higher the density, the brighter, or more luminous, the light emitted. Because of this property, mitochondrial density can be measured using the rhodamine-123 dye and luminosity data from collected images.

The mechanism for the movement and positioning of mitochondria, and other non-diffusible cellular components involves motor proteins, and the cytoskeletal filaments, actin microfilaments, and microtubules. Studies have shown that microtubules are actively involved in the trafficking of most larger structures, such as lysosomes (Hollenbeck, 1990). The motility of mitochondria is mostly the result of their travel along microtubules (Morris, 1993). The mitochondria can move along the microtubules at anywhere between 0.24 and 0.69  $\mu\text{m/s}$ , depending on the region of the cell and the direction of movement (Morris, 1993).

## Methods and Materials

## **Materials**

Nikon Eclipse E400 Microscope with fluorescence capabilities

Diagnostic Instruments Spot Insight QE Camera, with Spot Advanced 3.5 software

Macintosh G4 with OS X 10.2

ImageJ Image analysis software

Transfer pipettes

Small Petri dishes

37 degrees Celsius incubator

Corning glass slides

Coverslip fragments

Laminin/poly-lysine treated, ethanol washed and baked cover slips, with 17-hour growth sympathetic chick neurons from an 8 day old chick at a 4x density (generated from 30 trypsin-treated sympathetic neurons and 8 trypsin-treated dorsal root ganglia spread over 21 cover slips)

Hank's Balanced Saline Solution (HBSS)

F+ growth medium

1 ug/mL Rhodamine-123 in F+ growth medium

Vaseline/ paraffin wax mixture

## **Methods**

### **Staining of the mitochondria and coverslip preparation**

The treated coverslips were treated 17 hours after the plating with 1 ug/mL of Rhodamine-123 in the F+ growth medium for 10 minutes, at 37 degrees C, in darkness. After 10 minutes, the Petri dish containing the coverslip was removed from the incubator, and then washed three times in HBSS. Finally, the coverslip was re-suspended in a minimal amount of F+ growth medium. The coverslip was then removed from the medium carefully with positive-pressure forceps, the non-treated side of the coverslip was dried, and the coverslip was placed on top of a slide with a cover glass chip chamber, treated side down. The edges were then sealed with valap to seal the sample. Once dried, the top was washed with deionized water to remove any salt from the top of the coverslip. A control coverslip was created under the same conditions, except that the growth medium used for incubation was devoid of Rhodamine-123.

### **Imaging the cells**

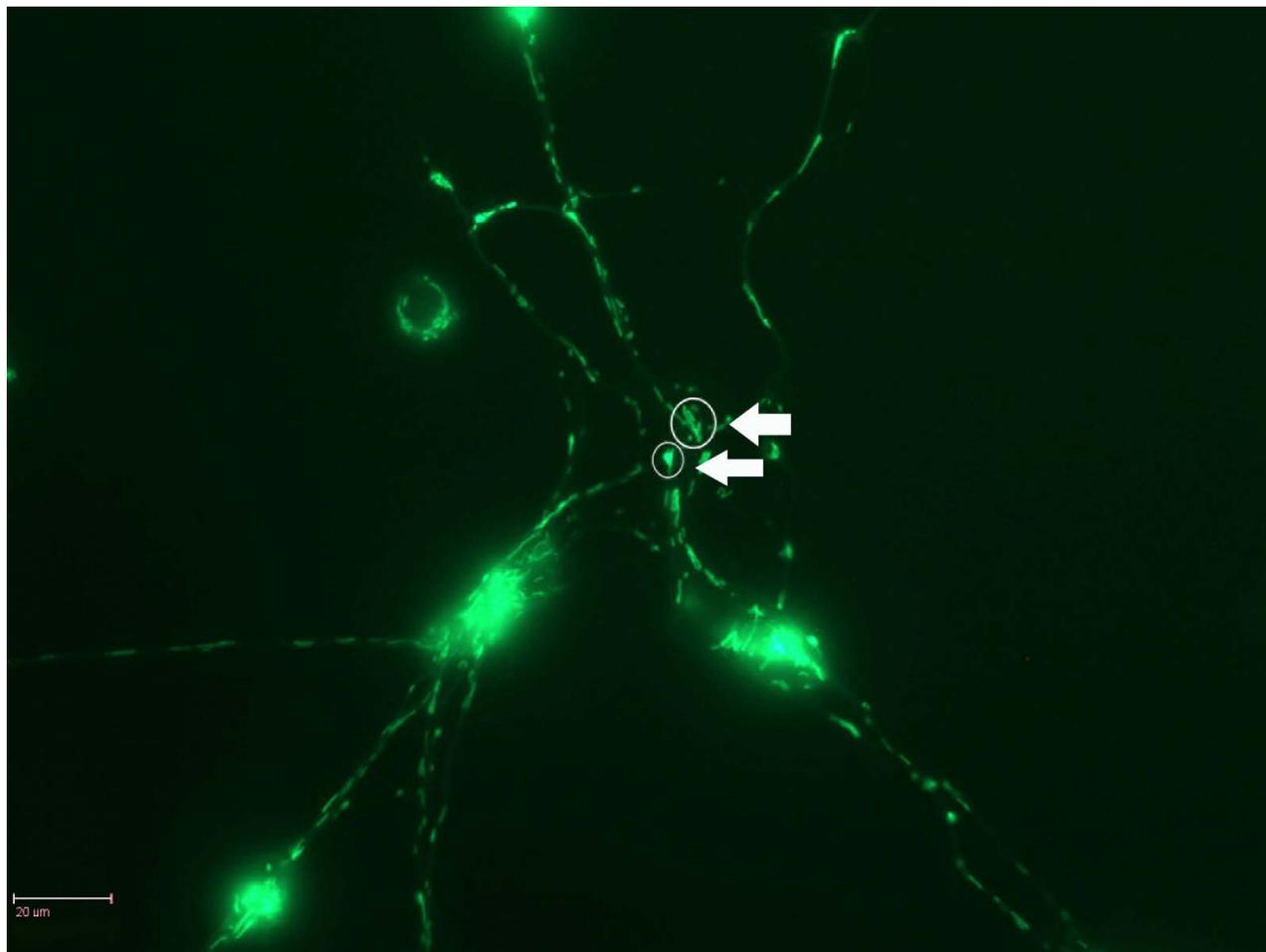
The cells were observed in brightfield, phase, and blue-emission fluorescence. Two images were taken for each area of interest--one in either brightfield or phase, and one under the blue-emission, green-emitting fluorescence. For the fluorescence, the following image settings were used to obtain the images:

400x magnification: 5000 msec exposure, 8 gain, 1.05 gamma

600x magnification: 2000 msec exposure, 1 gain, 1.05 gamma

### **Identifying target regions**

The regions of interest for this investigation were areas that appeared to be synaptic connections along an axon, ideally en passant synapses. In order to be certain that a region was just a synaptic overpass, where one axon went over the back of another, one axon in the ROI had to exhibit continuity in its membrane. These regions were identified on the images by looking for axonal areas that were visibly crossing another axon, and were not part of the same axon. Figure 1 holds examples of crossing axons and same axons, labeled. For each different image, after the experimental regions were identified, control regions had to be specified. The control region would serve as a baseline for comparison between the experimental regions of the cell. For each image, the invariable baseline was the cell body. A new baseline needed to be established in each image due to the varying levels of photobleaching present when the image was taken. If one cell body were used as a baseline for all images, the proportions would be distorted due to the inherent decrease in brightness over time of the fluorescent dye.



**Figure 1** -The above image has sample regions of interest (ROI) circled

## Analyzing the collected images

Once the images were collected, and the regions of interest identified, a mean luminosity of the region needed to be identified. To determine this, a region containing the mitochondria of interest was selected, with some background present, so that no region of any mitochondria would be left out. A histogram was then generated in ImageJ, and the number of pixels at each brightness level was exported to Microsoft Excel. This was done for all regions of interest, and the cell body, being sure to include some background all around in the region outlined. The data for each image was kept separate. Then, a histogram of a region of background was generated for the background, and that brightness data was exported, and then subtracted from the brightness data for each area of interest. For example, if the background had a brightness of between 15 and 22, for each data set for that image, all values 22 and under were considered background and disregarded. From the remaining data, the mean luminosity, in arbitrary units, was determined through simple averaging.

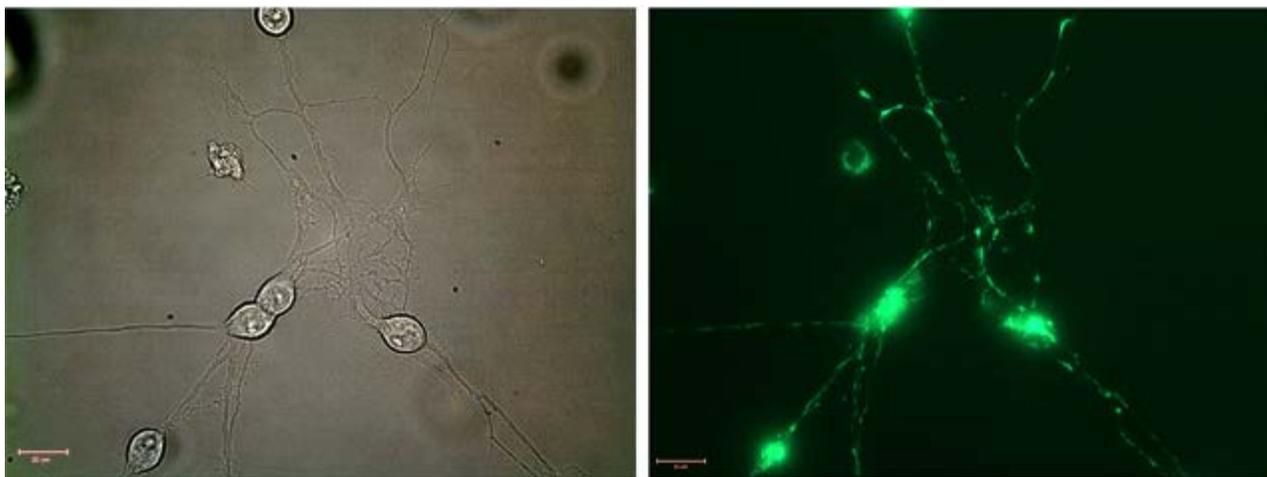
## Results

### *Procedural*

The imaging settings described previously in the methods produced very useable images, with very clear and bright images, as demonstrated with the representative example shown as Figure 3. The staining protocol worked well enough with the prescribed concentration of Rhodamine-123 and the desired incubation time, as evidenced by the brightness of the mitochondria, and the background was also slightly bright, but not bright enough to wash everything out, suggesting that the amount of washes used was also enough to remove adequate amounts of dye from the coverslip without removing all the cells from the substratum. The cellular density at the time of experimentation was very good for finding regions of interest in the cells that met the specifications set forth. The control used, a coverslip treated in exactly the same manner as the experimental coverslips, except for the incubation in the absence of Rhodamine-123,

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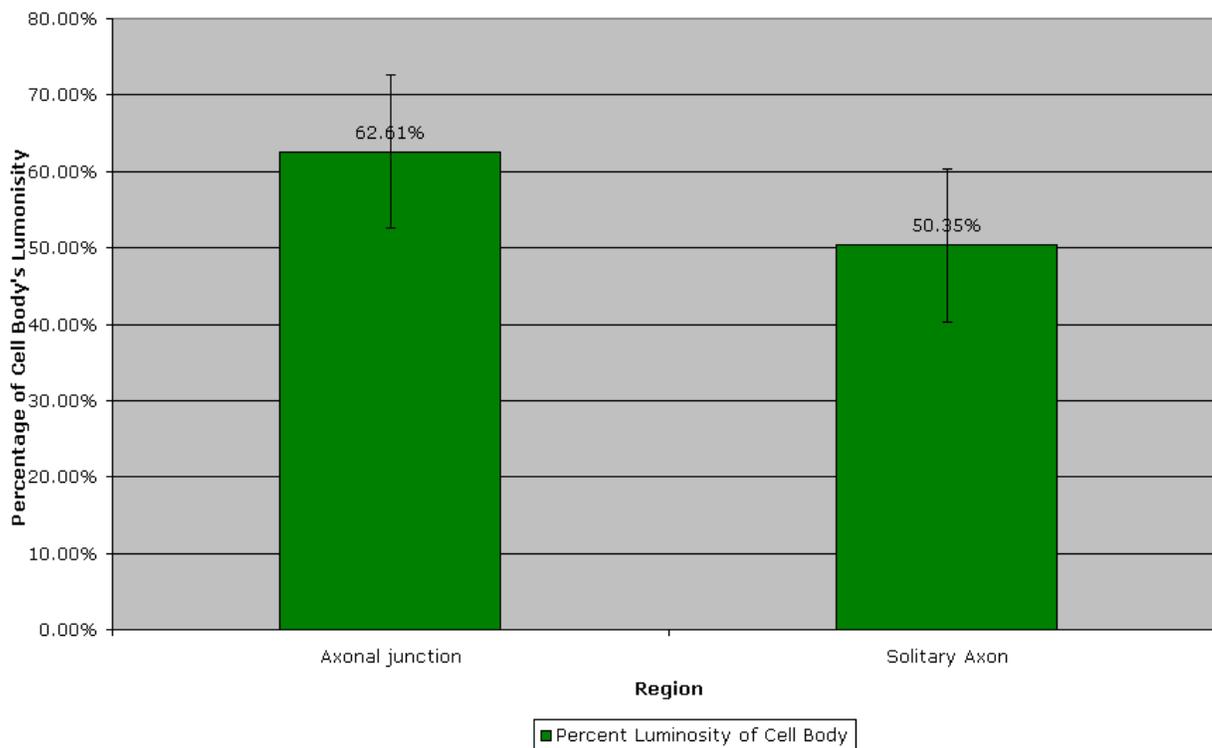
expressed no fluorescence under the same imaging conditions as the experimental coverslips.



**Figure 2** -This figure is a side-by-side display of brightfield and fluorescence images of the same cellular regions, taken at 600x. These images are representatives of all images taken. (Click image for fullsize version)

After taking images at both 400x and 600x magnification, the images used for data analysis were only those taken at 600x. This was because the 60x objective lens available on the E400 had a superior capability to pick up light, and produced better quality images, with less noise than was possible with the 40x objective.

### Percent Mean Luminosity of Cellular Regions Versus Cell Body



**Figure 3** -A graph comparing the mean luminosity of mitochondria in regions of axonal overlap to that of the mean mitochondrial luminosity (MML) of mitochondria in a non-overlapping, isolated region of an axon. The MML of each region was then made into a percentage of the MML of the cell body, and graphed.

### Analysis results

After interpreting the data collected from the images, a MML for each ROI (region of interest) was determined image by image. Due to the varying degrees of photobleaching and previous exposure, the MMLs could not be compared between images. Instead, they were compared to a cell body in their respective images. This generated a percentage luminosity of the cell body for each ROI on each image. With a baseline established, the percentage luminosities could then be compared between images for an overall MML for each ROI over the entire data set. Figure 3 depicts the relationship between the MML of overlapping axonal regions compared to the luminosity of the cell body, compared to the MML of a non-overlapping region of an axon, compared to the cell body. The MML of the axonal overlaps was consistently a higher percentage of the MML of the cell body than that of the non-overlapping axonal regions.

## Discussion

It has been previously shown that mitochondria congregate heavily in regions of a cell with high demands for energy (Wagner, 2003). Synaptic regions are areas of high endocytosis and exocytosis, both being high-energy activities for the cell. This tendency for mitochondria to tend towards areas of high energy demand has been demonstrated in neurons, particularly at the growth cones of growing neurons (Morris, 1993). Therefore, if a region of axonal overlap is synaptic, it should be expected that the mitochondrial density would be higher than in non-overlapping axonal regions.

The data collected in these trials currently support the hypothesis that areas of axonal overlap would have higher concentrations of mitochondria. This increase in mitochondrial concentration versus other regions of the axon suggests that there is an increased demand for energy at the regions of axonal overlap. This increased demand for energy might be due to a large amount of exocytosis and endocytosis in that region, which requires significant amounts of energy, and would indicate that the region is likely a synapse of some type, as any non-electrical synapse must be exocytosing a signaling molecule to its target cell. If these data were to be correlated with those obtained from a study of endocytic activity in the same cellular region, a more definitive conclusion on the reason for the enhanced mitochondrial presence in those regions could be obtained. For instance, if a study were to show that in regions that were identified by the same criteria used in this study a high amount of endocytosis was present compared to the rest of the axon, it would help further prove that these regions of axonal overlap can be, and often are, synaptic.

An additional experiment that would make for an interesting way to support or refute the data collected here would be the use of Quantum Dots as a fluorescent marker. Unlike organic stains, the Quantum Dots do not photobleach, and will remain optically active for much longer than most, if not all, fluorescent dyes. If this marker were coupled with a factor that would target it specifically to the mitochondria, so that the mitochondria would be labeled similarly to how they are labeled with Rhodamine-123, the persistence of the fluorescence could be used to take time-lapse images of the regions of interest, and their mitochondria. It may be that the mitochondria seen in this study were only present at the sites they were observed to be at for a short while; however, this was difficult to determine since the stain used deteriorated too quickly to make a comparison. With the Quantum Dots, a time-lapse image series could be generated, and from that series one could easily tell if the mitochondrial density changed drastically over time, or if it remained steady, to be ready for any high-energy-demand situation that might arise.

## References

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