CHAPTER

11 Digital quantification of neurite outgrowth and retraction by phase-contrast microscopy: A tau perspective

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Abstract

The proper organization and function of the mammalian nervous system relies on neuronal processes or "neurites," extended morphological projections that include axons and dendrites. Tau is a structural microtubule-associated protein that is widely expressed in the nervous system that mediates the establishment of cell polarity, neurite outgrowth, and axonal transport. A useful model for studying the establishment and maintenance of these neuronal structures are rat neuronal PC12 cells, which can be induced to express tau and project neurites by treating the cells with nerve growth factor. Here, we present a simple method for continuously measuring the rate of neurite outgrowth and retraction over time by neurite length and neurite area analyses. This method uses freely available ImageJ software and widely available phase-contrast imaging.

1 INTRODUCTION

The microtubule-associated protein tau is an essential protein that promotes the highly specialized morphologies in the nervous system and has important roles in cell differentiation and polarization. Primarily an axonal protein in mature neurons (in contrast to the dendritic MAP2), tau promotes the organized stabilization of microtubules and is a key regulator of neurite formation during neuronal differentiation (Drubin, Feinstein, Shooter, & Kirschner, 1985). As an intrinsically disordered protein, tau does not adopt a compact folded structure but rather is highly flexible and mobile (Schweers, Schönbrunn-Hanebeck, Marx, & Mandelkow, 1994). This flexibility allows tau to stabilize microtubules by binding to the MT surface and promoting self-assembly from tubulin subunits (Gustke, Trinczek, Biernat, Mandelkow, & Mandelkow, 1994; Witman, Cleveland, Weingarten, & Kirschner, 1976). As a result of its effects on microtubule biochemistry, tau regulates the rate of neurite elongation and stability (Butner & Kirschner, 1991; Drubin et al., 1985; Esmaeli-Azad, McCarty, & Feinstein, 1994). Axon outgrowth is inhibited when tau is suppressed using synthetic antisense oligonucleotides, which supports tau's necessity during axon outgrowth (Caceres & Kosik, 1990). Interestingly, when nonneuronal cells are induced to express high levels of tau, they can develop cellular processes containing dense arrays of bundled microtubules that are morphologically similar to neuronal axons (Baas, Pienkowski, & Kosik, 1991; Chen, Kanai, Cowan, & Hirokawa, 1992). Taken together, these studies suggest that tau's primary function is to promote microtubule assembly and bundling during neurite outgrowth.

The rat adrenal pheochromocytoma PC12 cell line has been a useful model for studying tau action during neurite outgrowth (Greene & Tischler, 1976). Upon treatment with nerve growth factor (NGF), PC12 cells cease to proliferate and extend branching processes similar to those produced by primary neurons in culture (Drubin et al., 1985; Greene & Tischler, 1976). Treatment of PC12 cells with NGF, either alone or together with dibutyryl cAMP (dbcAMP), causes a rapid

and strong induction of tau expression (Gunning, Landreth, et al., 1981; Gunning, Letourneau, et al., 1981). Tau expression is induced within 3–4 days of NGF treatment, coincident with a massive increase in microtubule assembly and neurite outgrowth. Upon NGF withdrawal, tau is quickly degraded while neurites disappear through retraction and fragmentation (Drubin et al., 1985). The ability to induce tau expression through NGF-mediated differentiation makes the PC12 line a valuable tool for investigating mechanisms of tau biology specifically and axonal homeostasis in general.

Here, we present a simple method for digitally quantifying neurite length and neurite area during differentiation of PC12 cells by phase-contrast imaging. Our methods, which should be generally adaptable to neurite outgrowth studies, take advantage of freely available ImageJ plugins combined with noninvasive phase-contrast imaging to measure neurite morphology continuously over time. The example data presented here specifically apply to neurite outgrowth; however, this method could also be used to study neurite retraction following the application of agents that perturb tau and/or microtubule stability.

2 MATERIALS

- Corning Costar 6-well or 12-well cell culture plates, flat bottom with lid, tissue culture treated, nonpyrogenic, polystyrene, sterile, 6-well Cat. 3516, 12-well Cat. 3513
- Gibco antibiotic–antimycotic 100× (contains 10,000 units/mL of penicillin, 10,000 µg/mL of streptomycin, and 25 µg/mL of Gibco Amphotericin B), Thermo Fisher Cat. 15,240,062, store at −20°C
- Dulbecco's-modified Eagle Medium powder, high glucose, [+] L-glutamine, [+] pyridoxine hydrochloride, [+] 110 mg/L sodium pyruvate, [−] sodium bicarbonate, Gibco 12,800–082, store at 4°C
- Bovine calf serum, supplemented, HyClone, Cat. SH30072.03, store at −20°C
- Heat Inactivated (HI) Horse Serum, Gibco, store at −20°C
- Poly-L-lysine hydrobromide, Sigma P2636-100 mg, in sterile water at 10 µg/mL, store at 4°C
- Sterile pipettes and Pipetteman or glass Pasteur pipettes with rubber bulb
- N^6,2′-*O*-dibutyryladenosine 3′,5′-cyclic monophosphate sodium salt (dbcAMP), Sigma D0260–100 mg. Make a stock solution of 250 mM in sterile DMSO, store 20 µL aliquots at −20°C. The final concentration is 0.5 mM (1:500 dilution from stock)
- NGF-2.5S from murine submaxillary gland, lyophilized powder, suitable for cell culture, Sigma N6009-4X25UG, diluted to 10 µg/mL in PC12 complete media, store 20 µL aliquots at −20°C
- Inverted brightfield microscope equipped with phase optics, camera, and 10× objective

3 METHODS

3.1 CELL CULTURE AND DIFFERENTIATION

1. Maintain PC12 cells on plastic dishes in Dulbecco's-modified Eagle's medium, supplemented with 5% supplemented calf-serum, 5% horse serum, 3.7 g/L $NaHCO_3$, and 1 × antibiotic/antimycotic at 37°C and 5% CO_2 until 50%–75% confluent (4–7 days for a 10 cm^2 dish).
2. Coat plastic cell culture dishes to be used for neurite outgrowth assays with 10 μg/mL poly-L-lysine (PLL) in sterile water for at least 1 h at room temperature in a cell culture hood prior to plating cells.
3. When ready to seed cells, aspirate as much of the PLL solution as possible and rinse the surface three times with enough 1 × PBS to cover the bottom of the well at room temperature, leaving the last wash in the well to prevent drying of the surface. With each wash, swirl the plate to collect any unadhered PLL (soluble PLL is toxic to cells). Set the plate aside while preparing cells.
4. Remove 80% of the media from the maintenance culture dish and discard.
5. Dislodge the cells from the surface of the maintenance culture dish by spraying the remaining media across the surface of the well using an automatic Pipetteman equipped with a 5-mL sterile pipette (alternatively, a Pasteur pipette with a rubber bulb can be used).
6. Triturate the cell suspension by drawing the cell suspension into the pipette and forcing it out against the bottom of the dish while holding the pipette opening flush against the bottom of the well. The cells are quite hardy and will not break from this treatment. Repeat 10 times until all the cell clumps are dissociated.
7. Determine the cell density using a hemocytometer. No tryphan blue is required if counting is done using a phase-contrast microscope.
8. Based upon the cell density, calculate the volume of cell suspension to add to fresh media so that the final cell density will be $1–5 \times 10^3$ cells per cm^2 of total surface area of either 6- or 12-well cell culture plates.
9. Add an appropriate volume of the cell suspension to the total volume of media needed for seeding all dishes or wells.
10. Invert the diluted cell suspension tube five times to homogenize the suspension.
11. Aspirate the PBS wash from each well of the PLL coated plate and immediately add the appropriate amount of diluted cell suspension to each well.
12. Return plate to incubator at 37°C and 5% CO_2 for 1–3 h to allow cells to adhere to the surface.
13. After 1–3 h, apply dbcAMP in DMSO to 0.5 mM final concentration to each well from a 250 mM stock solution in DMSO. This serves to initiate rapid neurite outgrowth.
14. Return the plate to the incubator at 37°C and 5% CO_2 for 24 h.
15. After 24 h, add β-NGF (dissolved in complete PC12 media) to a final concentration of 10–50 ng/mL to each well.

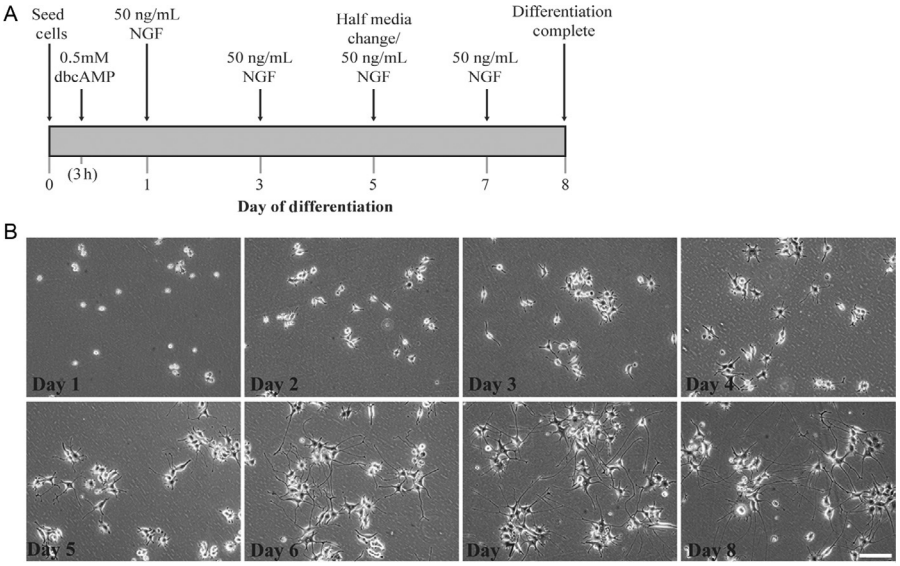

FIG. 1

PC12 differentiation by nerve growth factor and dbcAMP over 8 days in 5% HI-horse serum and 5% calf serum-containing media at 37°C and 5% CO_2. (A) Schedule of cell culture conditions. dbcAMP is added 3 h postseeding, then NGF is added to existing media 24 h later and every other day following. A half media change can be performed on day 5 if necessary, but may not be required. (B) Representative 10× phase-contrast images of PC12 cells corresponding to each day of the schedule shown in (A) scale bar = 100 μm.

16. Spike the media in each well every other day with NGF for the duration of the differentiation process (Fig. 1A).
17. On day 5, perform a half media change and supply fresh NGF.
18. If media begins to become acidic (orange color), perform a complete media change and supply fresh NGF.
19. Cells are considered fully differentiated after 7 days of NGF treatment (8 total days of differentiation). Cells should be imaged each day of the time course for subsequent analysis (detailed later).

Notes:

- It is essential that cells are plated at a density that is low enough for neurites to be able to project without contacting other cells or neurites early in the time course. If neurites encounter other neurites, they will stop their outgrowth and this will bias neurite outgrowth measurements.
- Cell dissociation can be monitored under a microscope. If cells are still significantly clumped when plated for differentiation, increase the number of trituration cycles to get as close to single cells as possible.

- Add NGF to each well in a sufficiently large volume of complete media to minimize pipetting error. The rate of neurite outgrowth is sensitive to NGF concentration.
- If a neurite retraction experiment is performed, it is essential to include an appropriate negative and/or vehicle control for any experimental condition. Ideally each multiwell plate seeded with the same cell dilution should have its own negative control on the same plate. The negative control allows each multiwell plate to be internally controlled for variations in cell seeding density and differentiation efficiency.
- Store plates being coated with PLL at 4°C overnight if unable to seed the same day. Allow the plates to come back to room temperature in a cell culture hood for ~30 min before plating cells, or place in an incubator at 37°C for 10 min.

3.2 PHASE-CONTRAST IMAGING

1. Be sure the microscope's condenser is properly aligned for Köhler illumination to achieve even illumination of the sample and to provide maximum contrast between cell bodies and neurites. To do so, bring the sample into focus under a 10 × objective, then fully close the field diaphragm, so that the edges of the field diaphragm are visible while looking through the eyepieces. While continuing to observe the opening through the eyepieces, adjust the height of the condenser so that the edges of the field diaphragm are sharp and well defined, then adjust the position of the field diaphragm opening using the adjustment screws so that is directly centered in the field of view. From the fully closed position, slowly open the field diaphragm until the edges of the opening are just outside the field of view.
2. Adjust the microscope's bulb intensity and camera exposure time, so that the background is a medium gray and the cell bodies are not overexposed (Fig. 1B). If possible, store the acquisition settings for future use.
3. Adjust the focal plane so that the neurites are in focus. The cell bodies may appear slightly out of focus but this will not affect analysis.
4. Image at least five random fields of view per well of each culture plate (Fig. 1B).

Notes:

- Even illumination is critical for quantitative phase-contrast microscopy. Thus, it is very important to minimize background interference and create as much contrast as possible between the background and neurites without overexposing the cell bodies. This can be achieved by ensuring the condenser is properly adjusted, the field diaphragm closed as much as possible, and the exposure properly set to adequately expose the cell's features.
- The periphery of most culture plate wells tends to have higher background and less contrast when using phase contrast. The best contrast can be achieved at or near the center of each well.

- Do not adjust the exposure settings during imaging. This will ensure that the background levels are consistent throughout the experiment and will produce higher quality neurite area measurements.

3.3 NEURITE LENGTH MEASUREMENTS

1. If the NeuronJ plugin (Meijering et al., 2004) is not already installed in ImageJ (Rasband, 1997–2016), install it by downloading the install files to ImageJ's *plugins* folder. Restart ImageJ after installing.
2. Once in Image J, open the NeuronJ plugin by selecting "NeuronJ" under the "Plugins" drop-down menu.
3. Open an image using the "Open" function under the "File" drop-down menu (Fig. 2A).
4. Ensure that the scale is correctly set for the image by selecting "Set Scale" under the "Analyze" drop-down menu. Enter the known distance in pixels per micron that corresponds to the objective used. This value must be determined for each individual microscope and camera setup using a calibrated slide. In our case, the 10× objective corresponds to 1.553 pixels/μm. Change the unit of measurements to "μm." Check the "Global" setting to apply this scale to all subsequently loaded images. Select "OK" to apply the scaling.
5. Click on the "Add tracings" button on the menu bar.
6. For each neurite to be measured (criteria below), click once at the origin of the neurite at the cell body and follow along the neurite's length with the cursor. If the automatic detection does not accurately trace the neurite, click along the length of the neurite to lock the tracing at certain points along the neurite. When the end of the neurite is reached, a double click on the mouse completes the tracing.
7. When all eligible neurites have been measured within the image, click the "Measure Tracings" button on the menu bar to measure the tracings. The individual tracing lengths and a summary of all tracings within the image can be copied to a spreadsheet or graphing software for analysis.
8. Save the tracing file to the same directory as the image. When the image is opened again within NeuronJ, the tracing file will be loaded automatically (Fig. 2B).
9. Before closing the image, count the number of cell bodies present. Use the "Multipoint Tool" found on the ImageJ menu bar to click on each cell body and record the final count to a spreadsheet.

Notes:

- Only neurites that originate and terminate in the same field of view should be analyzed. Any neurites that enter from outside the image or extend out of the image should be omitted from neurite length measurements.
- Neurites that cross other neurites should be excluded, as they may be stabilized as a result of their interactions.

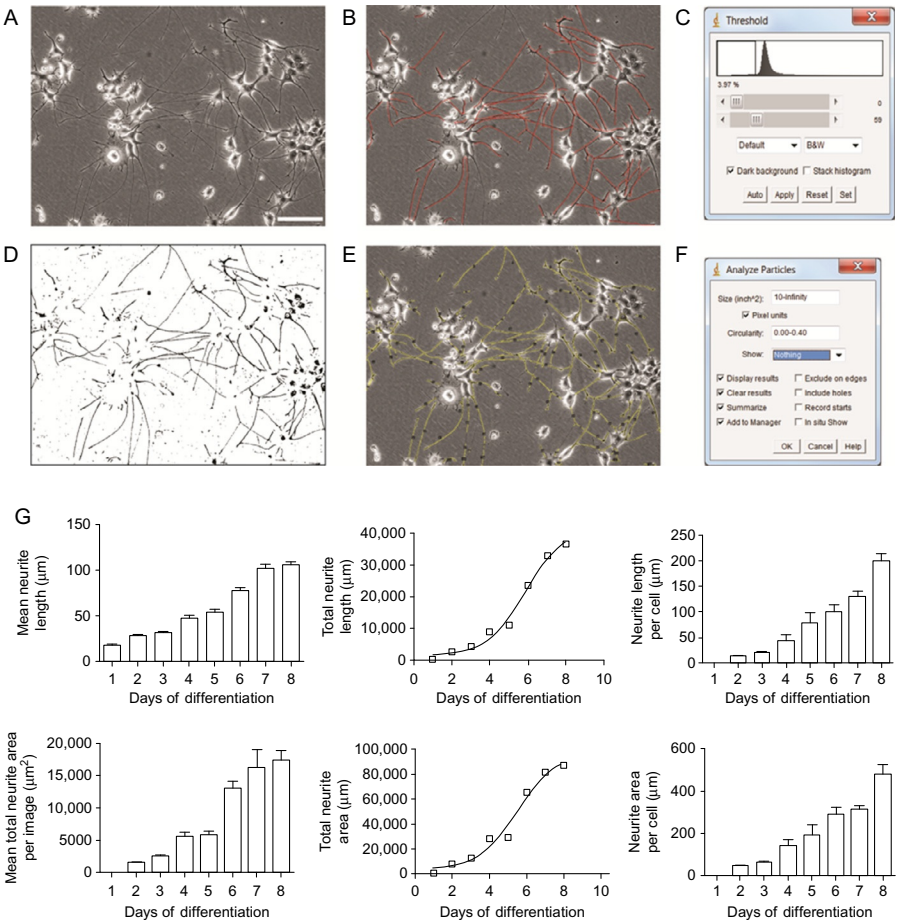

FIG. 2

Digital quantification of neurite outgrowth in differentiated PC12 neuronal cells. (A) Representative 10× phase-contrast image of day 8 PC12 cells, scale bar = 100 µm. (B) Neurites traced for length measurements (*red*) in the NeuronJ plugin for ImageJ. (C) Threshold settings used for neurite area measurements. Note the position of the minimum and maximum threshold values and "dark background" selection. (D) Thresholded version of the original image shown in (A). (E) Overlay of the original image and the identified areas from the thresholded image (shown in *yellow*). (F) Analyze particle settings, showing the particle size and circularity values. (G) Representative data of neurite length and neurite area over 8 days of differentiation. Graphs represent the mean neurite length or area (*left*), the total neurite length or area (*middle*), and the average neurite length or area per cell (*right*). Measurements are from five images per time point, and bar graphs are mean ± SEM.

- A good rule of thumb for determining what constitutes a neurite is that a "neurite" is at least as long as the diameter of the cell body.
- Holding the "Shift" key produces a straight line segment that is useful for navigating along neurites that may be close together. Inaccurate tracings can be removed by using the "Delete tracings" function on the menu bar.
- The available data in NeuronJ's measure function includes the length of each individual neurite, a sum of all lengths within an image, and mean neurite length.

3.4 NEURITE AREA MEASUREMENTS

1. Open ImageJ and load a 10 × phase-contrast image file. Ensure that the image is scaled correctly as described earlier.
2. Open the threshold settings under "Image," "Adjust," and "Threshold."
3. Drag the upper threshold bar to the lower limit, so that the number to the right displays "0." Ensure that the "Dark Background" box is selected and "Stack Histogram" is *not* selected (Fig. 2C). The neurites should appear black and the background white. Drag the lower threshold bar until the background starts to be visible (as black), then make small adjustments, so that the background is still white. The upper limit is typically set slightly to the left of the main peak shown on the threshold graph (Fig. 2C). Small specks in the background are acceptable and will be filtered out later. The image should be shown as thresholded as in Fig. 2D. Close the threshold window (do not select "auto" or "apply" from this window).
4. Select the "Analyze Particles" option under the "Analyze" drop-down menu.
5. Enter "10-Infinity" in the "Size" box, check the box for "Pixel units," enter "0.00–0.40" in the "Circularity" box, and then press OK (Fig. 2F).
6. A results dialog will open after "OK" is pressed. Notice which areas were selected in the image. If the identified areas are not appropriate, close the results windows without saving and adjust the analyze particles size and circularity values until only the neurite areas are identified.
7. Once appropriate particle size and circularity settings are determined that identify only neurites, they should be applied to all images in the set without adjustment.
8. Copy the results for individual particles from the "Results" window and from the "Summary" window to a spreadsheet. Repeat steps 1–5 for at least five images per condition, making sure to copy the results to a spreadsheet after analyzing each image.

Notes:

- If necessary, remove erroneous regions of interest (ROIs) by right-clicking the entry in the results window and selecting "Cut." Each ROI is numbered for identification. The ROIs can also be overlaid onto the original image to verify neurite identification. To do so, open the original image again through ImageJ

without closing the thresholded and analyzed image. Under the "Image" drop-down menu, select the "Overlay" menu and select "From ROI Manager" (Fig. 2E). If desired, the ROIs can be saved for each image by selecting "More" from the ROI Manager and selecting "Save." Load existing ROIs by selecting "Open" from the same menu.

- The particle size and circularity settings listed here are given as starting points and should be optimized for the level of background in the image (particle size filter) and general shape of cell bodies of the neurons being analyzed (circularity filter). A larger circularity value will maintain particles that are more round (i.e., cell bodies) while a smaller value will maintain more longer and straight particles (neurites).
- Each image must be manually thresholded and the limits should be similarly set to achieve consistent analysis throughout the data set. Thus it is important during image acquisition to maintain a consistent background intensity.

3.5 DATA ANALYSIS

1. Data are processed in Excel and then transferred to GraphPad Prism or similar software for statistical analysis and graphing. Several parameters are quantified. The mean neurite length in each image is calculated by dividing total neurite length by the number of neurites. Data from at least five images are averaged to get the mean neurite length for each experimental condition (Fig. 2G, top left). The total neurite length for each experimental condition (the sum of the lengths in all five images) is also a useful measurement, as some conditions may have near-zero neurite length (Fig. 2G, top middle). The mean neurite length per cell in each image is calculated by dividing the total neurite length by the number of cells, and data from at least five images are averaged to get the mean neurite length per cell for each experimental condition (Fig. 2G, top right).
2. Similar analyses are performed with neurite area data to obtain the mean neurite area per image, total neurite area, and mean neurite area per cell (Fig. 2G, bottom).
3. Before comparing across experiments, each plate should be normalized to its own control so that neurite measurements are internally controlled for variations in cell seeding density and growth conditions. It is therefore important to include a negative and/or vehicle control on each multiwell plate to use for normalization. For example, divide the total neurite length of each experimental condition by the total neurite length of the control. Normalized data are expressed as a fraction or percent of control.

4 CONCLUSIONS

We presented a simple method for digitally quantifying neurite length and area during differentiation of PC12 cells. This method allows continuous measurement of neurite morphology during neurite outgrowth and can also be applied to neurite

degeneration in live cells. In principle, this method could be applied in studies involving rates of axon outgrowth as well as mechanisms of neurotoxicity resulting from insults to neuronal homeostasis.

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