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Objective Morphological Quantification of Microscopic Images Using a Fast Fourier Transform (FFT) Analysis

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Abstract

Quantification of immunohistochemistry (IHC) and immunofluorescence (IF) using image intensity depends on a number of variables. These variables add a subjective complexity in keeping a standard within and between laboratories. Fast Fourier Transformation (FFT) algorithms, however, allow for a rapid and objective quantification (via statistical analysis) using cell morphologies when the microscopic structures are oriented or aligned. Quantification of alignment is given in terms of a ratio of FFT intensity to the intensity of an orthogonal angle, giving a numerical value of the alignment of the microscopic structures. This allows for a more objective analysis than alternative approaches, which rely upon relative intensities.

Keywords

quantification; microscopy; fast Fourier transformation; immunohistochemistry

OVERVIEW AND PRINCIPLES

The staining intensity of immunohistochemistry (IHC) depends on multiple factors, such as the concentration of both primary and secondary antibodies, incubation periods, temperature, tissue quality, and reagent purity. Microscopy settings and the perception of individuals viewing the images adds additional variability. These factors create an unnecessary variability in the inter-laboratory, and even intra-laboratory, quantification of IHC. Regardless, quantification of IHC is routinely reported based on relative staining intensity.

2-D Fast Fourier Transformation (FFT) has been used to quantify microscopic structures related to elastin networks in the tunica media of blood vessels (Tonar et al., 2003). The FFT represents the pixel intensity of the original photomicrograph in the frequency domain (Fourier space) instead of a spatial domain, producing a grayscale FFT image of the frequency content. When adjacent pixels in the original photomicrograph define a straight

line, the FFT plots a straight line through the origin (Tonar et al., 2003). The summation of pixel intensities in radial coordinates around the origin produces a line graph with two peaks (180° apart), corresponding to the original angle of alignment. Commercial and freeware image-processing software can apply the FFT rapidly to any image (Alexander et al., 2006). The information in the FFT image can be quantified by measurements of the radial sum intensity for 360 radii around the center of the FFT image. Radial sum intensities peak at the primary angles of alignment, and the degree of orientation can be defined as a ratio of the peak intensity to the intensity at an orthogonal angle. A photomicrograph with no alignment will show a constant pixel intensity absent of peaks, indicating no specific directionality.

PROTOCOLS

Basic Protocol 1: Preparation of Images for FFT Analysis

In the following example, we have imaged tissue prepared by subjecting adult rats to diffuse brain injury and contracting Neuroscience Associates Inc. (<http://www.neuroscienceassociates.com>) to section and stain brain injured and control brains for the Iba-1 marker of microglia, as previously published (van Bregt et al., 2012). Digital photomicrographs were taken using bright-field microscopy and a 20× objective lens.

FFT analysis of the photomicrographs first requires a 1000 × 1000 pixel circle feather mask to be applied separately to each photomicrograph (1360 × 1024 pixels). The benefits of a feather mask are twofold: (1) eliminating the edge effect of the rectangular photomicrograph, and (2) improving the radial sum intensity that gives rise to the morphological analysis. The 1000 × 1000-pixel circle feather mask for this method was created in Adobe Photoshop CS5 software.

Materials—Histological sections on microscope slides (e.g. brain sections as described in van Bregt et al., 2012)

Research microscope with attached digital camera

Image processing software (e.g., Adobe Photoshop)

Acquire images

1. Place the slide on the microscope stage, making sure that the dorsal-ventral midline (or similar anatomical landmark) of the tissue section is aligned with one edge of the digital image preview.
2. Locate the tissue section of interest and center the area of interest with the digital image preview.

When capturing images for analysis, the “angle of interest” should not be 90° in the horizontal or vertical planes, as this may interfere with later analysis.

IMPORTANT NOTE: The captured photomicrograph must be saved in TIFF format to reduce the possibility of erroneous results.

3. Note the alignment of the microscopic structures. Standardize the alignment between slides. Acquire the image.

Lock the exposure settings between sections and slides to maintain uniform exposure between images. For our analysis using the Olympus AX80 research microscope and Olympus DP controller software, the exposure can be locked using the “AE lock” key under the exposure mode.

Prepare image for FFT analysis

4. In Photoshop, create a new image from the File tab with a width and height of 1000 pixels and a resolution of 72 pixels per inch. Select for an 8-bit RGB color image in the Color Mode drop-down option.
5. Select Window > Tools to open the toolbar, if it is not open by default. Left-click and hold the Rectangular Marquee Tool to select the Elliptical Marquee Tool. Alternatively, you can press M (or shift-M) on the keyboard. In the options bar (located near the top of the screen), define the style as Fixed Size with dimensions of 1000 pixels by 1000 pixels. Click and drag in the center of the image to identify and place the circle marquee inside the image. Use the arrow keys on the keyboard to center the circle in the image.
6. In the Tools toolbar, select the Paint Bucket tool. If not immediately evident, left-click and hold the Gradient Tool to select the Paint Bucket Tool. Alternatively press G (or shift-G). Once selected, select Windows > Color and choose the foreground color as “black”.
7. Select Windows > Masks, allowing you to feather the image. The Pixel Mask option may have to be selected on the Masks toolbar. Set the feather options to 20 pixels and 100% density.
8. Once the 1000 × 1000-pixel circle feather mask is finished, save as a new image. This file can be used repeatedly to apply a circle feather mask to all images. See Figure 9.5.1.
9. Open the desired photomicrograph, duplicate the layer, and delete the original (background) layer using the Layer tab.

This enables editing, as the original layer is locked.

10. Duplicate 1000 × 1000-pixel circle feather mask onto the photomicrograph. With the Feather Mask file active, select Layer > Duplicate Layer. Name the new layer as feather mask in the appropriate box and select the filename of the photomicrograph from the drop-down list. Ensure that the 1000 × 1000-pixel circle feather mask is applied in the same location of every photomicrograph.
11. In the layers window of Adobe Photoshop, click, hold, and drag the 1000 × 1000-pixel circle feather mask (white circle on black background, the image on the right of the “feather mask” layer) onto the photomicrograph layer. Proceed by deleting the former “feather mask” layer. Flatten the image by right-clicking on the layer and selecting Flatten Image.

12. Save the current image as a new TIFF file.

Ensure that the 1000 × 1000-pixel circle feather mask image file is the same size as the photomicrograph. This allows the feather mask to be placed in the same location on every image.

Basic Protocol 2: FFT Analysis of Images

FFT analysis allows the morphological quantification of microscopic structural alignment. The pixel information of the photomicrograph is transformed into Fourier space (power spectra). The radial sum intensities (constrained within the ring analysis) are generated for 360 radii around the center of the FFT image. Pixel intensity values are calculated for each point (angle) around the circumference of the ring centered on the FFT image.

Materials—NIH Image J software with the “Oval profile Plot” plug-in (author, Bill O’Connell, <http://rsb.info.nih.gov/ij/plugins/oval-profile.html>)

Spreadsheet calculation software (e.g., Microsoft Excel)

1. Open a saved photomicrograph (with the feather mask applied) in NIH Image J software. See Figure 9.5.1C.
2. Using the “angle tool” of the main toolbar, estimate and note the angle of interest for the anatomical structures from the vertical. Single-click at the start of the aligned microscopic structure of interest, then at the end of the structure, and then on a point vertical and down from the origin.

This angle will be used as reference to the true angle of alignment from the histogram produced later. See Figure 9.5.2A.

3. Select “process > FFT > FFT” from the menu bar to generate the FFT image, whereby the photomicrograph is transformed into its sine and cosine components and the pixel information is mapped in terms of relative intensities along specific orientations.

A new image window will open in NIH ImageJ.

4. Apply a ring analysis to the FFT image using the “oval tool” (in the main toolbar) and a defined radius. Holding down Shift while using the oval tool ensures creation of a symmetrical circle. Ensure the ring captures the entirety of the FFT starburst without including unnecessary dead space. See Figure 9.5.2A. Make note of the size of the ring to ensure consistency across all photomicrographs.

The center of the ring should overlap the center of the FFT image; this can be done by using the arrow keys of the keyboard. In our lab, we use a radius of 700 pixels.

5. Select “plugins > Input-Output > Oval profile” from the menu bar. Set “number of points” to 360; “Analysis mode” to radial sums; deselect the box marked “Show hotspots”.

This step will sample the specified number of angles (360 in our experiments) around the oval described. A radial sum intensity graph will be generated in a separate window.

IMPORTANT NOTE: *The plugin must be installed.*

- a. To install, you must first download and save the Oval Profile plugin in the ~ImageJ/Plugins/Input-Output folder.

See materials list above for ImageJ URL.

- b. Open ImageJ (restart ImageJ if already open); the Oval Profile plugin can now be found in Plugins > Input-Output > Oval Profile.
6. In the newly generated radial sum intensity graph window, click on “list” to view the radial sum intensity values for all 360 angles. Highlight and copy the values to a new spreadsheet.
7. Plot all values into an x - y scatter graph.

The two peaks of radial sum intensity across the angles represent the angle of interest and the opposite angle of interest; the two troughs of the radial sum intensity represent the orthogonal angle and the opposite orthogonal angle. The angles at the peaks represent the alignment angle of the microscopic structures; this should be similar to the estimation of alignment angle previously calculated using the angle tool in NIH ImageJ. See Figure 9.5.2.

8. As microscopic structures are not perfectly aligned, samples of the radial sum intensities around the four angles are taken into consideration as well as the angles themselves. In our lab, we use ± 5 angles around each of the angles. This provides radial sum intensities at 22 angles for the angle of interest (+opposite angle of interest) and radial sum intensities at 22 angles for the orthogonal angle (+opposite orthogonal angle).
9. The ratio to the mean orthogonal angle represents the morphological quantification of alignment in microscopic structures. The equations for the ratio to mean orthogonal angle are below. Also see Figure 9.5.3.

$$\text{a. } RMS = \sqrt{\sum_{i=1}^{360} A_i^2 / 360}$$

$$\text{b. } Ave_M = \sum_{j=1}^{22} \left(\frac{M_j}{RMS} \right) / 22$$

$$\text{c. Ratio to the mean orthogonal angle} = \left\{ \left[\sum_{k=1}^{22} \left(\frac{N_k / RMS}{Ave_M} \right) / 22 \right] \times 100 \right\} - 100$$

where:

RMS = Root mean square of all radial sum intensities;

A = The radial sum intensities of all 360 angles.

M = The radial sum intensities of 22 angles, including the orthogonal angle ± 5 flanking angles and the opposite orthogonal angle ± 5 flanking angles.

N = The radial sum intensities of 22 angles, including the angle of interest ± 5 flanking angles and the opposite angle of interest ± 5 flanking angles.

10. A value of 0 for the ratio to the mean orthogonal angle represents no alignment for the microscopic structures; a value of 100 represents complete alignment for the microscopic structures. For an annotated Microsoft Excel worksheet, see Figure 9.5.4. A sample spreadsheet is available for download as supplementary material.
11. For reproducibility, our laboratory repeats the whole measurement process three times per image, using three images from separate sections per animal. Use appropriate statistical tests for your study.

IMPORTANT NOTE: If large peaks are seen in the scatter plot at values 0° , 90° , 180° , and 270° , these artifacts are due to the axes on the FFT image and should be deleted for presentation purpose in all spreadsheets.

COMMENTARY

Background Information

FFT allows for an objective quantification of photomicrographs based on morphology, which reduces the subjectivity associated with pixel-intensity methods. This method of morphological quantification is based upon on the assumption that the cell morphology in the original photomicrograph represents linearity. When this assumption is correct, the alignment can be transformed into the frequency domain, generating an FFT image with a perpendicular line plotted through the origin. The applied ring analysis extracts data from the frequency domain of the FFT image and provides radial sum intensity values for 360 radii. A scatter plot of the radial sum intensity values versus angle shows peaks corresponding to the angle of interest and the opposite angle of interest, and troughs at the orthogonal angle and the opposite orthogonal angle. The radial sum intensity values for the four angles of interest (± 5 flanking radii for each angle) were used to calculate a value of alignment. A photomicrograph with no alignment will show a constant pixel intensity, and mathematically give a value of 0.0 for alignment. As this method assumes alignment in the photomicrograph, a value > 0.0 for a photomicrograph with a constant pixel intensity is more likely.

The above method of morphological quantification can be used for any given photomicrograph, as long as the assumption of alignment is met. Further applications of FFT quantification can be found in Figures 9.5.4 and 9.5.5. Rat brain tissue was positively stained by immunohistochemistry for oligodendrocytes (anti-CNPase) and neurons (anti-pan Neuronal) within the cortex and thalamus of the brain. FFT analysis was performed on the photomicrographs as described. Alignment for both cell types was observed in the cortex of the sectioned tissue; values for the ratio to the mean orthogonal angle were calculated at 8.7 and 12.6 for oligodendrocytes and neurons, respectively. There was either nominal or no alignment observed in the thalamus; values for the ratio to the mean orthogonal angle were calculated at 1.9 and 0.8 for the oligodendrocytes and neurons, respectively.

The advantages of FFT include the speed with which the analysis can be performed, reproducibility and standardization between immunohistochemistry runs, and the identification of subtle alignment that may be overlooked. FFT can be used on biological tissues that show alignment on a straight line, such as the cell cytoskeleton, blood vessels, neuronal circuitry, and connective and muscle tissue fibers. Disadvantages of FFT include not being able to perform the analysis on microscopic structures that align but do not describe a straight line. The quantitative results serve to supplement the information in original images.

The following citations provide published examples of analyses that incorporate FFT analysis (see Table 9.5.1). Tonar et al. (2003) discuss background and methodology of FFT in detail, elaborating on some of the more difficult to understand aspects of FFT. The idea of using NIH ImageJ image-processing software for the analysis of microscopic structures is given by Alexander et al. (2006). Valmikinathan et al. (2008) explain the interpretation of the radial intensity histogram, and Ayres et al. (2006, 2007, 2008) further describe useful applications of the method above.

Critical Parameters and Troubleshooting

1. Select an objective magnification with appropriate resolution to capture individual microscopic structures.
2. If the cell population of alignment is at either 0° or 90° , rotate the microscope stage or the images in an image-processing program by 45° . This is to ensure that the FFT axes seen above in Figure 9.5.2 do not interfere with the radial sum intensity values.
3. To maximize the information obtained by the FFT analysis, the background color of the mask and/or the inversion of the photomicrograph may need to be taken into consideration. For example, it may be more efficient to use a white mask for dark stained tissue on a white background. The reverse is true, using a black mask for an image with a white foreground and black background.
4. Although the reference angle is measured and verified, the ratio calculation is independent of the angle of interest. This calculation allows for comparison between measurements.

Anticipated Results

The ratio to the mean orthogonal angle assigns a numerical value to the observed alignment. For example, Figure 9.5.2 shows an alignment of 42.0 for steel wire, 28.0 for bamboo, 7.8 for aligned brain microglia, and 0.3 for resting brain microglia. These values are only partially comparable, as the steel wire and bamboo are given as examples of possible structures that could be used in FFT analysis. These images have not been captured with the same microscope image-acquisition settings. The digital photomicrographs, however, have been captured with the same image acquisition settings, and therefore are comparable. The ratio to the mean orthogonal angle for the photomicrographs is greater for aligned cellular structures (Fig. 9.5.2C, 9.5.5Ai, and 9.5.5Bi) than structures that do not show alignment

(Fig. 9.5.2D, 9.5.5Aii, and 9.5.5Bii). The quantitative values represent the alignment seen in the images themselves.

Replicate measurements are necessary to reduce variability. In our lab, triplicate measurements were taken for (1) the number of animals in each group to reduce the biological variability between animals; (2) brain sections at similar locations, to ensure that the observation is not unique to a single tissue section; and (3) FFT analysis for each photomicrograph, as the placement of the feather mask in Adobe Photoshop software and the Oval in NIH ImageJ software includes some subjectivity.

Time Considerations

The time considerations are independent of the tissue preparation and staining procedures. For image acquisition, one should schedule 1 to 3 hr, depending on the number of images to be acquired; 1 hr is approximate for 50 images. High-quality images, with standardized acquisition settings, are highly encouraged. Once the 1000×1000 -pixel circle feather mask has been created, image preparation and FFT analysis takes less than 10 min per photomicrograph to conduct the analysis in triplicate. One study on 15 animals, three photomicrographs per animal, and three sets of image preparation and FFT analysis per photomicrograph took our lab approximately three 3-hr sessions after IHC had been performed.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Literature Cited

- Alexander JK, Fuss B, Colello RJ. Electric field-induced astrocyte alignment directs neurite outgrowth. *Neuron Glia Biol.* 2006; 2:93–103. [PubMed: 18458757]
- Ayres CE, Bowlin GL, Henderson SC, Taylor L, Schultz J, Alexander J, Telemeco TA, Simpson DG. Modulation of anisotropy in electrospun tissue-engineering scaffolds: Analysis of fiber alignment by the fast Fourier transform. *Biomaterials.* 2006; 27:5524–5534. [PubMed: 16859744]
- Ayres CE, Bowlin GL, Pizinger R, Taylor LT, Keen CA, Simpson DG. Incremental changes in anisotropy induce incremental changes in the material properties of electrospun scaffolds. *Acta Biomaterialia.* 2007; 3:651–661. [PubMed: 17513181]
- Ayres CE, Jha BS, Meredith H, Bowman JR, Bowlin GL, Henderson SC, Simpson DG. Measuring fiber alignment in electrospun scaffolds: A user's guide to the 2D fast Fourier transform approach. *J. Biomater. Sci. Polym. Ed.* 2008; 19:603–621. [PubMed: 18419940]
- Tonar Z, Nmeek S, Holota R, Koová J, Teká V, Moláček J, Tomáš Kohoutek H. Microscopic image analysis of elastin network in samples of normal, atherosclerotic and aneurysmatic abdominal aorta and its biomechanical implications. *J. Appl. Biomed.* 2003; 1:149–159.
- Valmikinathan CM, Tian J, Wang J, Yu X. Novel nanofibrous spiral scaffolds for neural tissue engineering. *J. Neural Eng.* 2008; 5:422. [PubMed: 18971515]

van Bregt DR, Thomas TC, Hinzman JM, Cao T, Liu M, Bing G, Gerhardt GA, Pauly JR, Lifshitz J. Substantia nigra vulnerability after a single moderate diffuse brain injury in the rat. *Exp. Neurol.* 2012; 1:8–19. [PubMed: 22178300]

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Figure 9.5.1.

Stepwise progression to prepare the feather mask on a digital photomicrograph. (A) Original photomicrograph. (B) The 1000×1000 -pixel circle feather mask layered on top of the photomicrograph (Basic Protocol 1, step 7). (C) Finished photomicrograph after the feather mask has been applied to the photomicrograph and layers flattened (Basic Protocol 1, step 8).

Figure 9.5.2.

FFT analysis for a variety of structures that show alignment. (A) Demonstrates the FFT analysis for steel wire aligned at 135° from the vertical. The alignment is seen as a 'starburst' effect at the center of the Fast Fourier Transformation (FFT) image, which has predominant signal at angles congruent with the original image (major peaks at 135° and 315°). The radial sum intensities for 360 angles around the center of the FFT image are plotted as an x-y scatter graph. Prominent peaks are seen at 135° and 315°, in accord with the original image and FFT image. The minor peaks at 90°, 180°, 270°, and 360° are from

the axes of the FFT itself. For illustrative purposes, the orange horizontal line represents the root mean square of the radial sum intensities; the purple and cyan vertical lines represent the angle of interest and opposite angle of interest, respectively; the red and green vertical lines represent the orthogonal and opposite orthogonal angles, respectively. **(B)** FFT analysis for bamboo, with similar major peaks as the steel wire. The minor ‘starburst’ peaks at 45° and 225° are congruent with the orthogonal angle and opposite orthogonal angle, resulting from the bamboo notches (confirmed by digital removal of the notches; data not shown). Both **(A)** and **(B)** are examples of alignment that may be seen in a cell population. **(C)** FFT analysis performed on Iba-1 stained microglial population that shows alignment. The cell morphology of the population is aligned at approximately 144° from the vertical. **(D)** FFT analysis performed on Iba-1 stained microglia population that shows no alignment, as indicated by the horizontal line graph.

	Angles of Interest		Orthogonal Angles		Ratio to Mean Orthogonal Angle = [nRMS Angle / AVERAGE(nRMS Orthogonal Angles)] -100
	Raw = VALUE	nRMS = VALUE/RMS	Raw = VALUE	nRMS = VALUE/RMS	
Angle of Interest	88061.2	1.040	88622.0	1.050	7.3%
	87952.5	1.040	89092.6	1.050	7.2%
	88656.1	1.050	88564.1	1.050	8.0%
	88656.7	1.050	88723.9	1.050	8.0%
	88268.8	1.040	88881.2	1.050	7.5%
	88258.8	1.040	88815.2	1.050	7.5%
	87973.6	1.040	89068.5	1.050	7.2%
	87588.2	1.040	89364.6	1.060	6.7%
	88191.5	1.040	87945.5	1.040	7.4%
	88261.2	1.040	88410.5	1.050	7.5%
	88418.5	1.050	88838.1	1.050	7.7%
Opposite Angle	81987.8	0.970	82833.8	0.980	8.0%
	82102.7	0.970	82891.0	0.980	8.5%
	81469.8	0.960	82541.1	0.980	7.9%
	81986.9	0.970	82030.8	0.970	8.1%
	81852.2	0.970	82604.0	0.980	8.3%
	82185.3	0.970	82251.8	0.970	8.2%
	81559.4	0.960	82443.7	0.970	8.5%
	81757.5	0.970	82180.1	0.970	8.9%
	81572.5	0.960	82588.2	0.980	7.1%
	81097.9	0.960	81885.9	0.970	7.7%
	81588.3	0.960	82422.6	0.970	8.2%
RMS: 85868.9 = SQRT(SUMSQ(All 360 Raw Values)/360)					Ave: 7.8%

Figure 9.5.3.

Screenshot of the datasheet and formulas used to calculate the ratio to the mean orthogonal angle for a single photograph seen in Figure 9.5.2C.

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Figure 9.5.5.

FFT analysis for two different anatomical structures found within the brain of an adult male rat. **(A)** FFT analysis and radial sum intensity values of CNPase-stained oligodendrocytes located within the cortex (i) and the thalamus (ii). Cortical oligodendrocytes can be observed to align at an angle of 125° from the vertical. A value for the ratio to the mean orthogonal angle was calculated at 8.7 in this photomicrograph. Nominal alignment can be observed for the thalamic oligodendrocytes. A value for the ratio to the mean orthogonal angle was calculated at 1.9. **(B)** FFT analysis and radial sum intensity values of pan-neuronal-stained

neurons located within the cortex (i) and thalamus (ii). Cortical neurons can be observed to align at an angle of 120° from the vertical. A value for the ratio to the mean orthogonal angle was calculated at 12.6 in this photomicrograph. No alignment was observed for the thalamic neurons. A value for the ratio to the mean orthogonal angle was calculated at 0.8.

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Table 9.5.1

Previously Published Examples of FFT Analysis of Photomicrographs Used in Experimental Models

Model	Species	Tissue	Software	Stain	Analysis	Reference
Normal, atherosclerotic and aneurysmatic aorta	Human	Smooth muscle, elastin and collagen fibers of the aorta	MATLAB (The MathWorks) ^a	Hematoxylin and eosin	FFT	Tonar et al. (2003)
Fiber alignment on electrospun scaffolds of gelatin	—	Natural and synthetic polymers	NIH Image J	—	FFT	Ayres et al. (2006, 2007, 2008)
The influence of astrocytic alignment on neurite outgrowth	Rat	Dorsal root ganglia	NIH ImageJ	GFAP (mature astrocytes), vimentin (immature astrocytes), and TUJ1 (neurons)	FFT	Alexander et al. (2006)
Fiber alignment in electrospun nanofiber scaffolds	Rat	Peripheral nerves	NIH ImageJ	—	FFT	Valmikinathan et al. (2008)

^a <http://www.mathworks.com/>.