

Video Article

Optical Scatter Microscopy Based on Two-Dimensional Gabor Filters

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Abstract

We demonstrate a microscopic instrument that can measure subcellular texture arising from organelle morphology and organization within unstained living cells. The proposed instrument extends the sensitivity of label-free optical microscopy to nanoscale changes in organelle size and shape and can be used to accelerate the study of the structure-function relationship pertaining to organelle dynamics underlying fundamental biological processes, such as programmed cell death or cellular differentiation. The microscope can be easily implemented on existing microscopy platforms, and can therefore be disseminated to individual laboratories, where scientists can implement and use the proposed methods with unrestricted access.

The proposed technique is able to characterize subcellular structure by observing the cell through two-dimensional optical Gabor filters. These filters can be tuned to sense with nanoscale (10's of nm) sensitivity, specific morphological attributes pertaining to the size and orientation of non-spherical subcellular organelles. While based on contrast generated by elastic scattering, the technique does not rely on a detailed inverse scattering model or on Mie theory to extract morphometric measurements. This technique is therefore applicable to non-spherical organelles for which a precise theoretical scatter description is not easily given, and provides distinctive morphometric parameters that can be obtained within unstained living cells to assess their function. The technique is advantageous compared with digital image processing in that it operates directly on the object's field transform rather than the discretized object's intensity. It does not rely on high image sampling rates and can therefore be used to rapidly screen morphological activity within hundreds of cells at a time, thus greatly facilitating the study of organelle structure beyond individual organelle segmentation and reconstruction by fluorescence confocal microscopy of highly magnified digital images of limited fields of view.

In this demonstration we show data from a marine diatom to illustrate the methodology. We also show preliminary data collected from living cells to give an idea of how the method may be applied in a relevant biological context.

Video Link

The video component of this article can be found at <http://www.jove.com/video/1915/>

Protocol

1. Getting the cells ready

1. The cells that were plated the day before need to be labeled with Mitotracker green for fluorescence imaging of the mitochondria.
2. Remove 100 μ M stock solution of Mitotracker green in DMSO made previously from the 4°C freezer, and warm to room temperature with the hand. Also take out bovine aortic endothelial cells (BAEC) cell culture medium also previously prepared and warm to 37°C in the benchtop waterbath.
3. Once the Mitotracker and culture medium are warmed, place these into the hood making sure to sterilize your gloved hands and all outer surfaces of the containers with 70% ethanol solution. Do not turn on the hood light, as the fluorescent label is light sensitive and will quickly photobleach in ambient room light.
4. Making the right concentration for the mitochondrial labeling is very important. Too little won't label the mitochondria effectively, while too much Mitotracker can have toxic effects. A concentration of 100 nM of Mitotracker incubated for 45 minutes with the cells works well. Prepare this concentration by adding 100 μ L of Mitotracker stock to 10 mL culture medium in a 15 mL tube. This will make plenty for at least one experiment.
5. Replace the existing medium with the labeled medium by sucking out the old medium with a Pasteur pipette connected to the vacuum line. Then immediately add 2 mL of the labeled medium to each occupied culture well in the 6 well plate.
6. Because the fluorescent label is sensitive to light, replace the cells in the incubator quickly without exposing to direct room light. Covering the 6 well plate with the hands works well for this. The cells will stay in the incubator for 45 minutes.

2. Getting the optical setup ready

1. While the cells wait in the incubator, we have to turn on the optical setup. In the optics room, first turn on the mercury arc lamp, followed by the computer, the microscope, the cameras, and the laser. Then plug in the digital micromirror device (DMD) and the spinning diffuser.
2. Check to make sure that the optical launch is aligned by looking through the microscope eyepiece to ensure that the field of view is brightly illuminated by the laser light.
3. Clean the objective by folding one piece of lens paper into a tight square and grasp tightly with a hemostat. Dip the lens paper into ammonia-free glass cleaning solution to absorb a tiny amount into the paper. Rap the hemostat on your free hand several times to remove any excess. Wipe the objective by firmly applying one clean, continuous swipe across the objective from one end to the other, going over the lens in the middle. Do not re-swipe or scrub. Discard used paper.
4. To load the sample, place the graticule over the 63x oil immersion objective by dropping 1-2 small drops of immersion oil over the objective while the objective is all the way down. Then place the graticule in the stage. Then raise the objective so that the oil "grabs" the sample. Focus the sample in the eyepiece.
5. To align the condenser, adjust the condenser height so that it is aligned in central Kohler illumination by focusing the hexagonal edge of the condenser field stop. Center the condenser field stop over the field of view if necessary by turning the condenser centering knobs. The condenser aperture stop should be closed.
6. Start the IPlab program and input the settings to operate the RoperScientific Cascade 512 camera. Confirm that the camera is set to frame transfer mode. Start the live preview by running the "Acquire focus" command. Set the index prefix and file location to which the images will be saved.
7. Start the RSImage program and input the settings to operate the CoolSnap program. Clocking mode should be set to normal.
8. Start the DMD software and place the dark field iris on the script menu, followed by the "Load and Reset" command and run the script.
9. Send the light to the DMD and Cascade 512 camera by setting the microscope optovar and viewport to LSM. This will send the image through the DMD and the aligned optics, projecting the DF image onto the CCD. The dark field (DF) image should appear on the live preview already underway in IPlab. Adjust the fine focus of the microscope if necessary to focus the image on the live preview.
10. Take a snapshot of the field of view using the "acquire single" command. Set the exposure time high enough to ensure at least 10000 counts of signal in the image. After acquisition, use the "save as indexed" command to save the image to disk. This image of the graticule measures the size of the field of view (FOV).
11. Now, move the graticule sample so that the graticule is beyond the FOV so that only background is visible. Acquire a background image of the field at a long enough exposure time to ensure that at least 5000 counts of signal is acquired. This image will aid in background subtraction of the unfiltered images.

3. Loading the filterbank and using the setup to acquire filtered-background images

1. Now, we need to acquire Gabor-filtered images of the background. Load the Gabor filter bank script to the DMD control software. Run the entire script to buffer the filters to the onboard memory of the DMD; this might take a few minutes.
2. Once the entire script is buffered, we can now acquire filtered images of the background. Use the start and stop markers within the DMD software to instruct the DMD to load only one set of filters corresponding to one Gabor-like filter at a time, and run the script. The live image preview should change from dark field to the filtered image for that filter.
3. Open the acquisition script in IPlab from disk. Adjust the exposure time to ensure that at least 2000 counts of signal are being acquired. As the DMD script is running, cancel the live preview in IPlab and run the acquisition script. This will automatically acquire, index and save the filtered image to disk.
4. Once the first image is acquired, stop the script running in the DMD software and delete the used commands from the script. Replace the start and stop markers at the beginning and end of the next filter set. Repeat the acquisition in IPlab.
5. Repeat step 3.4 until the entire filterbank has been used and all filtered images have been acquired and saved.

4. Plating the cells

1. By now, the cells will soon be ready to plate for the experiment. Plug in the soldering iron on the lab benchtop. Remove the L15 viewing medium and heat to 37°C. Make a work station with a paper towel and a Kimwipe. Make several wicks by tearing and twirling Kimwipes. The wicks will help in transferring fluid to and from the cell plate.
2. After this, we need to plate the sample. We use the machined metal sample holders to plate our cells, making a "coverslip sandwich" with the metal plate in between. Apply a thin bead of vacuum grease from a syringe around the upper periphery of the metal plate hole extending about halfway to the ends of the grooves on each side. Gently press a clean no. 1 coverslip onto the grease. Flip the plate over and apply grease around the hole. Turn off the room lights.
3. Now we get the cells from the incubator, handling the incubator contents with nitrile exam gloves sterilized with 70% ethanol. Remove the cell plate from the incubator, holding your breath while the incubator door is open. Be careful to minimize the exposure to room light.
4. Remove the coverslip that will be used for the experiment from the six-well plate, noting that the side that was face-up in the well is the side with cells attached. Carefully dry the coverslip on both sides until it is almost completely dry, while keeping track of which side of the coverslip has the cells. Then press the coverslip, cell side down, into the greased metal plate over the viewing hole, making sure that no air gaps remain within the grease layer. The grease must form a watertight seal to allow for us to load the cells with the L15 medium. Once you are certain of this, flip the plate back over.
5. Pipette the L15 medium into the plated cells by forcing the liquid through the groove between the upper coverslip and the metal plate. Pipetting 200 μ L at a time works well. The first volume pipette should fill the space sandwiched between the coverslips with the liquid extending almost to the groove on the other side.
6. Pipette another 200 μ L of medium into the plated cells, but this time, hold a wick at the opposite groove so that the medium flows from one side to another. This washes the cells and removes any traces of the old medium. Be careful to prevent any bubbles from forming within the liquid during this step. Repeat this process 2-3 times using a new wick for each rinse.

- Remember the soldering iron we plugged in? Now is the time it gets used. Flip the plate upside down once more, supporting the plate from the edges so that liquid is trapped in the cell reservoir and cannot drip down. Dip the soldering iron into the valap beaker. This will quickly melt some of the valap which will then cling to the soldering iron tip. Carefully apply the molten valap around the edges of the bottom coverslip (which is now facing upward) using the soldering iron tip as an applicator. Continue dipping and applying until a you go all the way around the coverslip perimeter, sealing the coverslip to the metal plate.
- The bottom coverslip has cells growing on it, and may have residue from the dried up medium on the exposed side. Clean any residue from the coverslip surface by balling up a Kimwipe and cleaning the coverslip in a single sliding motion much like cleaning the objective. This ensures that the coverslip is cleanest in the center where it will be viewed.
- Unplug the soldering iron and return the 6-well plate to the incubator observing the same containment and sterility procedures. Take the plated cells to the optics lab and mount on the objective as described in steps 2.4 and 2.5.

5. Conducting the experiment

- Find a nice field of healthy-looking cells.
- Acquire a dark field image of the field of view. Align the microscope in differential interference contrast (DIC) and acquire a DIC image. Make sure that the exposure times are long enough to ensure that signal is adequate.
- Now we have to acquire the fluorescent images on the other camera. To get DIC images on the CoolSnap, we use a blue LED attached to the condenser, replacing it and removing it as necessary. While the microscope is still aligned in DIC, Send the light to the CoolSnap by setting the microscope optovar to 1.0x and viewport to 100% binocular. Divert the image from the eyepiece to the camera. Place the LED over the condenser to illuminate the field and preview the FOV in RSIimage and adjust fine focus if necessary. Acquire a DIC image and save to disk. Note how the FOV is different from the one obtained from the Cascade camera. These images will have to be registered during the analysis phase after the experiment.
- Obtain a fluorescence image by adjusting the filter cube to the fluorescein filtercube. Acquire an image by briefly turning on the fluorescence excitation using the microscope and then turn it off as soon as the acquisition is completed. Since we focused the sample in DIC, the fluorescence image is focused as well. This saves on exposure time in fluorescence, thereby slowing down photobleaching. Save the fluorescence image to disk.
- Now we have to acquire the filtered images. Reset the microscope to dark field and resend the light through the LSM port as in 2.9.
- Run the entire Gabor filterbank script as in 3.3-3.5. We have now completed the data acquisition for one time point.

6. Switching the medium to expose the cells to staurosporine (STS), and maintaining the medium throughout the experiment

- While the cells are still on the stage and without disturbing the field of view, switch out the regular L-15 medium for the same containing a 1 μM solution of STS made from a 4 mM stock solution of STS in DMSO. Use the wicking method described in steps 4.6 to switch the media.
- Now, we repeat steps 5.2-5.6 for subsequent time points. We repeat this process until the experiment is completed.
- During the course of the experiment, more medium will have to be added so the sample does not desiccate. This is accomplished by pipetting medium into the groove of the cell plate without removing from the stage and without disturbing the FOV.

7. Representative Results

At the conclusion of the experiment, the collected data will include a large number of filtered images that need to be processed to extract the subcellular structural data. Two examples are shown for an optical filter bank consisting of 9 Gabor-like filters with filter period $S=0.95\mu\text{m}$, Gaussian envelope standard deviation $s=S/2=0.45\mu\text{m}$, and orientations $\Phi=0^\circ$ to $\Phi=160^\circ$ in 20° increments. (See also [1] for more detail).

Example 1: Marine Diatom

We first applied our orientation sensitive filter bank to a marine diatom sample (Carolina Biological Supply Company) with oriented features that were clearly visible in dark-field (DF) imaging (Fig. 1). The optically filtered images are shown alongside the unfiltered image of the sample for comparison.

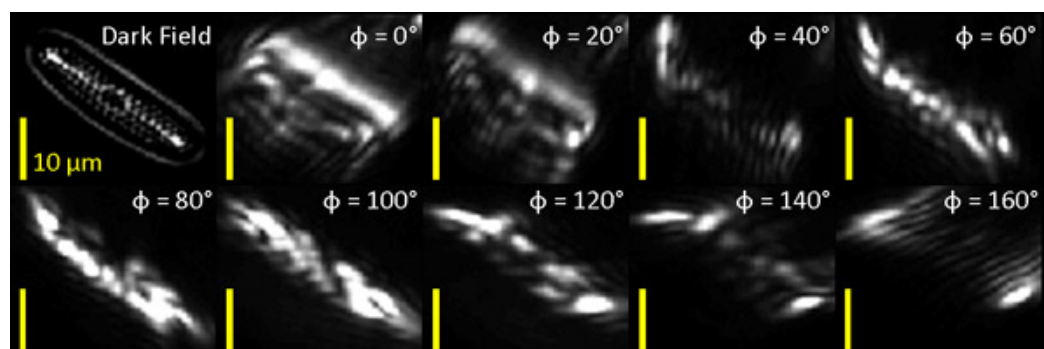


Figure 1: Dark field (DF) and optically filtered image of marine diatoms. We will analyze the diatom in the lower right of the image (white arrow in the left most panel).

The set of nine Gabor-filtered images of the diatom were processed pixel-by-pixel for object orientation and roundness. Processing consisted of (1) summing measured responses of all nine Gabor-filtered images at each pixel to determine the overall magnitude of the signal response thereby encoding response significance, and (2) finding the Gabor filter orientation, Φ , at which the response is maximized and taking the ratio of this maximal response to the average response for all angles thereby encoding the extent to which objects at each pixel have a preferred orientation. The degree of orientation is closely related to the geometric aspect ratio of the particle. In Fig. 2B, the overall response of the pixel to the filter bank (parameter 1) and the degree of orientation or aspect ratio (parameter 2) are encoded in the color saturation and hue, respectively. An aspect ratio near 1 (blue) is present in areas in which there is no preferred response angle, while greater values (red) indicate areas in which a higher preferred angle response is present. Substructure particle orientation is encoded in a quiver plot (Fig. 2C), where each line closely agreed with the underlying local object orientation visible in unfiltered dark-field (Fig. 2A).

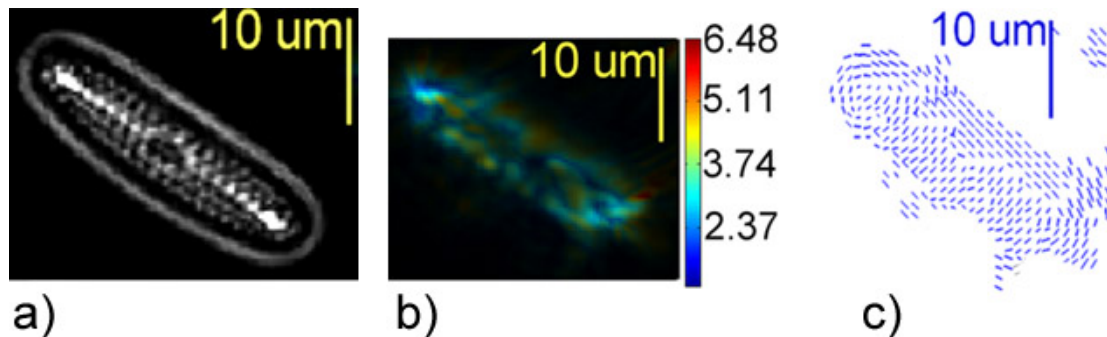


Figure 2: A: Dark field image of diatom. B: Object orientation image. Color scale indicates degree of orientation (aspect ratio) while brightness encodes significance of the total Gabor filter response. C: Orientation of objects with response intensity $\geq 10\%$ of maximum. Line segment indicates the corresponding structure's long axis.

Example 2: Apoptotic cells

Here we show filtered images of bovine endothelial cells treated with staurosporine (STS) that were processed in the same way as the diatom. Fig. 3 shows an unfiltered dark-field (DF) image of the cells along with the nine filtered images at time $T = -180$ min. prior to STS treatment.

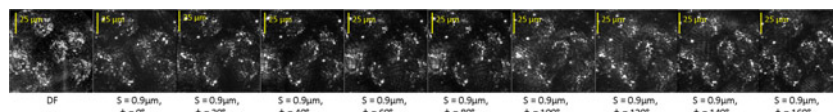


Figure 3: Dark field (DF) and optically filtered images of a field containing several living endothelial cells.

The filtered images were subsequently acquired every 20 minutes for a period of three hours after STS treatment. Fig. 4a shows an aspect ratio map of the cells as a function of time. In this case the color hue represents the degree of orientation (labeled orientedness) as for the color hue in Fig. 2b above. However, the aspect ratio brightness was not weighted by the average filter response. By registering our aspect ratio maps with fluorescence images of the labeled mitochondria in these cells (Fig. 4b), we determined that the measured aspect ratio drop was confined to the cellular regions containing mitochondria and was concomitant with mitochondrial fragmentation which could be observed directly in the fluorescence images of the same cells. Fig. 5 shows time plots depicting the change in aspect ratio as a function of time in cells undergoing apoptosis. Within each cell, there is a drop in aspect ratio at $T = 60$ -100 min in the regions that register with fluorescent mitochondria, but not in regions that register with the dim background fluorescence areas.

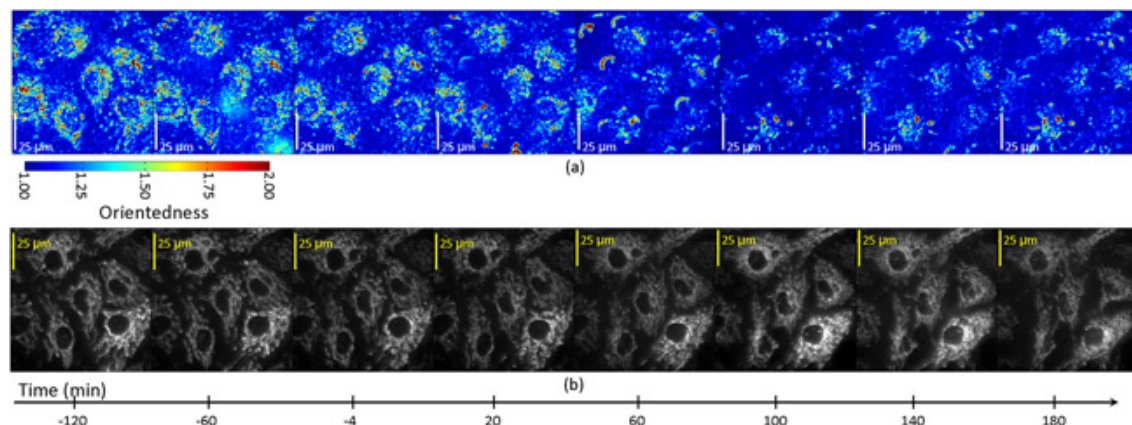


Figure 4: Aspect ratio (a) and fluorescence (b) images of endothelial cells treated with the apoptosis inducer, staurosporine.

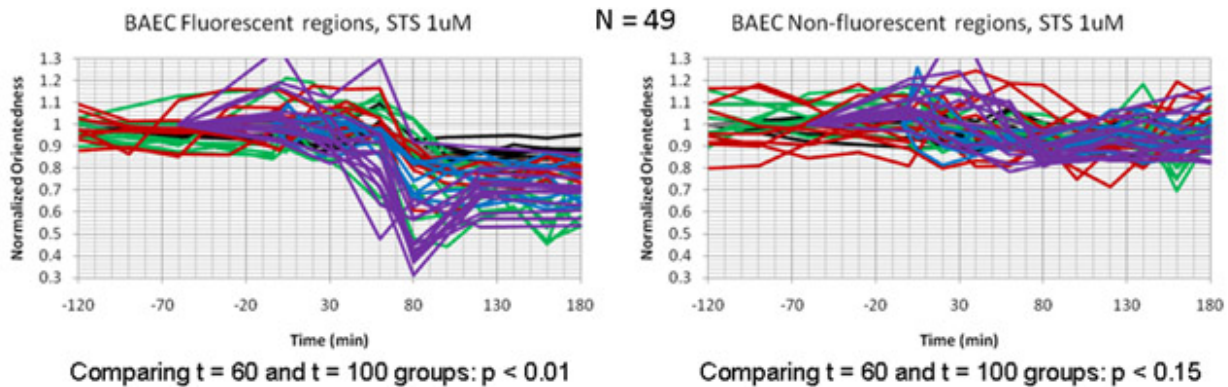


Figure 5: Time plots comparing the decrease in particle aspect ratio (orientedness) in endothelial cells treated with staurosporine. The individual traces represent time plots within single cells. The drop in orientedness is confined to the regions of the cells that register with fluorescent mitochondria (left panel) and is absent from the remaining background fluorescence regions (right panel).

Now that we have determined that the aspect ratio drop corresponds to mitochondrial fragmentation, we can induce apoptosis in these cells, measure the fragmentation using our optical scatter method without having to label the cells, and study the effect of different genetic and experimental conditions on this dynamic.

Discussion

The method described above yields morphometric maps of the object that may encode particle size or orientation for example. This structural information can be used in several ways:

1. It can be used as an initial screen to identify tissue or cell regions that are altered during a specific treatment and then further analyze these regions with specific molecular and biochemical assays.
2. It can be used in conjunction with fluorescence to yield a multimodal analysis of the cells that combines a simultaneous understanding of molecular activity (by fluorescence) with quantitative characterization of subcellular structure (by this method).
3. Once a specific structural response is correlated with a specific organellar activity in a specific biological process (e.g. apoptosis), the method can be used without any fluorescence labels to screen for different genetic and experimental conditions affecting this structural response.

To date we have shown that the method is sensitive to differences in particle size on the order of 30-50nm [2]. We have shown that the method is sensitive to changes in particle orientation and aspect ratio [3] and to a decrease in particle aspect ratio consistent with apoptosis-induced mitochondrial fragmentation (Figs. 4-5 above).

The results of example 2 suggest that our method permits dynamic measurements of cellular function that can be interpreted in terms of specific organellar function and that can be collected without fluorescence labels or exogenous dyes. However, initial validation of the measured responses against specifically labeled organelles was necessary. Once this initial validation is completed, organellar dynamics may be probed directly with our label-free method.

Applying the method to multiple cellular conditions, and correlating our dynamic structural measurement with the location of the different organelles within the cell, as we did with mitochondria, can ultimately lead to a library of dynamic structural behaviors that can uniquely characterize specific cellular states (e.g. apoptosis, oxidative and metabolic stress, inflammatory response etc). This information could further be incorporated into a "cell state analyzer", which can be used in a variety of applications including drug discovery in clinical cell analysis.

It is important to note that the method outlined here represents a general approach in which optical scatter data acquired in a microscopic imaging system can be used to extract specific subcellular dynamics. However, the specific instrumentation used can be significantly improved. In particular, the current choice of spatial light modulator for spatial filtering may not be optimal to maximize the efficiency of data acquisition, spatial frequency resolution, and optical signal throughput. Chromatic and geometric aberrations associated with the digital micro-mirror device used here are discussed in [3]. We are currently investigating the potential advantages and drawbacks of a state-of-the-art liquid crystal device in place of the DMD to mitigate these issues. In addition, the device for data acquisition, spatial filtering and microscope control are actuated separately by the human user. This greatly limits acquisition time where a large number of filtered images need to be collected before processing. Thus, automation of the setup is imperative to make the acquisition time commensurate with the duration of the CCD exposure, which is expected to reach 10's of milliseconds per filtered image for adequate signal-to noise. This increase in temporal resolution will also allow us to more precisely characterize the structural dynamics of organelles within living cells. We are therefore actively working on developing a customized graphic user interface that can unify the control of the hardware and streamline the actuation of its components, including microscope control, CCD image acquisition, and optical filtering at the spatial light modulator.

Disclosures

Some aspects of the methods have been included in and invention disclosure, Rutgers University Docket # 09-049.

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