Development of a Laser Gate for Protein Transport Analysis at the Ciliary Tip

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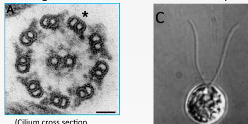
Abstract

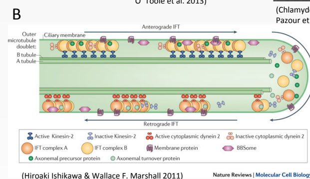
Cilia and flagella are cellular extensions that function in motility and sensing. Human sperm cells use cilia for locomotion and the multiple cilia of epithelial cells in the airways move foreign particles outward. Non-motile cilia in the eyes and nose function in the perception of light and chemical signals. Because cilia lack ribosomes, the RNA-protein particles responsible for protein synthesis, all proteins needed for cilia function or growth must be transported from the cell body into the protruding organelle. Intraflagellar transport (IFT) is one pathway for proteins transport from the base of the cilia, to the tip, and back again. Important steps of cilia assembly are thought to happen at the ciliary tip; cilia grow by the addition of subunits at the tip and IFT complexes are remodeled at the tip to allow their return to the cell body. The goal of my work is to develop an improved technique to image individual protein particles at the ciliary tip. Proteins of the IFT particles are made visible under the microscope via florescent protein (i.e. GFP) tagging. However, crowding of the particles at the ciliary tip largely obscures the visibility of individual proteins. A focused laser beam is used to bleach the fluorescence of some IFT particles at the tip. This increases the clarity for the remaining particles, but the tip is quickly refilled with florescent particles. We used a laser gate to control the bleaching laser; the laser blinks on and off at the base of the cilium in a pattern that will bleach most IFT particles but allows a few particles to pass unbleached through the gate, enter the cilium, and be imaged as individual particles while they complete their journey through the cilium. Further, we controlled the camera to not record while the bleaching laser is on; this will facilitate data analysis as it avoids recording of overexposed frames. This concept is carried out using a program written in Micro Manager and is communicated through an Arduino to the laser shutter. This program controls the laser shutter and the camera to capture movies of single IFT particles and their cargoes inside the cilia.

Introduction

Cilia are extracellular extensions that have many functions such as the motion of cells (i.e. human sperm cells) and sensing (i.e. light perception in the eye). Cilia lack ribosomes, the organelle responsible for protein synthesis, and all proteins necessary for growth and function of the cilia must be transported into the organelle. This is done via intraflagellar transport (IFT; B). In IFT, the protein cargo is loaded onto the IFT trains consisting of IFT motors and IFT A and B subcomplexes, and moves from the base of the cilia to the tip (anterograde IFT). At the tip, the IFT trains release the cargo and are remodeled to return back to the base by retrograde IFT. Due to the vast number and function of cilia in the human body, a defective cilia, such as an issue in IFT, can cause problems on a larger scale. A few examples of these ciliopathies are kidney disease, male infertility, or blindness. Using *Chlamydomonas reinhardtii* (C) as a model, we are able to visualize IFT transport using total internal reflection microscopy (TIRF) and florescent tags such as green florescent protein (GFP). The tip of the cilium is a place of interest due to the remodeling of the IFT complex and because cilia are believed to grow from the tip. However, due to the concentration of protein particles being transported into the cilia and the larger diameter of the fluorescence, it is difficult to clearly see individual particles, especially at the tip. Bleaching the particles via a focused laser beam is a method used to clear some fluorescence away. Bleaching will not eliminate or destroy the IFT particle but merely impair light emission from GFP making the particle invisible. After this bleach, the cilium quickly refills with fluorescing proteins. Therefore, it is necessary for the laser to flash on and off to keep too many fluorescing proteins from entering into the cilium. This is the concept of the laser gate.



(Cilium cross section, O' Toole et al. 2013)
(Chlamydomonas, Pazour et al. 2000)

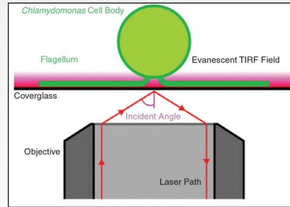


(Hiroaki Ishikawa & Wallace F. Marshall 2011)
Nature Reviews | Molecular Cell Biology

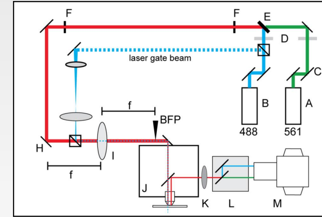
Methods

TIRF

Total internal reflection fluorescence (TIRF) microscopy is used to image the movement of protein particles in cilia. The laser beam is directed through the objective and reflects off the slide at the incident angle. This reflection creates the evanescent TIRF field of 30 – 300 nm in depth. Cilia have a diameter of ~200 nm, thus TIRF allows for imaging proteins in cilia with little interference from the cell body.

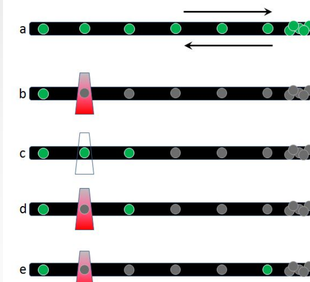


(from Benjamin D. Engel, Karl-Ferdinand Lechtreck, Tsuyoshi Sakai, Mitsuo Ikebe, George B. Witman, and Wallace F. Marshall)



A, 561 nm diode laser, 75 mW (Spectraphysics); **B,** 488 nm diode laser, 40 mW (Spectraphysics); **C,** stirring mirrors (Semrock), 25 mm; **D,** neutral density filter wheels (Newport) to adjust laser intensity; we used ND 1.0 and ND 1.5 (corresponding to 10% and 3% transmission, respectively) for the 488- and 561-nm lasers, respectively. **E,** shutters (to block the 561-nm or both lasers beams, respectively); **F,** LaserMUX Beam Combiner (Semrock), reflects the 488 nm lasers, lets the 561 laser pass through, 25 mm; **G,** zero aperture iris diaphragm (Thorlabs) for aligning the two lasers parallel; **H,** periscope to bring laser beam to the height of the microscope; **I,** steering mirror. Mirror in kinematic mount with three actuators (used to adjust the laser from epifluorescence to TIRF illumination); **J,** mounted 400-700 nm achromatic doublet lens, f 100 mm, (Thorlabs); **K,** Nikon Eclipse Ti-U with a GFP/mCherry TIRF filter cube and a 60x NA 1.49 TIRF objective; **L,** C mount with 2.5x lens (this set-up allows a large field of view to find suitable cells using the eyepieces and optimal magnification of the specimen for the resolution of the camera); **M,** Photometrics DualView2 Image Splitting Device; *N,* Andor iXon X3 DU897 EMCCD camera. The lens is positioned one focal distance (f) from the back focal plane (BFP) and the steering mirror is position 2x f behind the BFP.

Laser Gate



a. The protein particles tagged with GFP move in both anterograde and retrograde direction via IFT. There is crowding at the tip as the IFT particles dock and remodel.

b. The entire cilium is bleached by moving it through the laser spot (red) from tip to base.

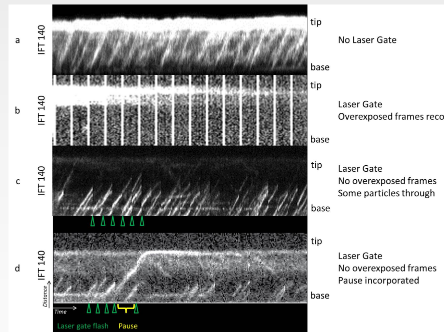
c. The laser is turned off and a single particle is let through.

d. The laser is turned back on. One particle has already made it through and the following particles are bleached by the flashing laser.

e. Since all other particles will be bleached, the unbleached particle can be clearly imaged as it continues its journey to the tip and back.

Program Development

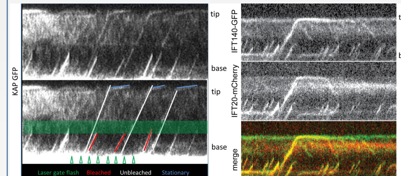
Using Micro Manager and an Arduino, we wrote a program to carry out the laser gate. Micro Manager is a microscopy software that allows custom control of the camera and shutter using BeanShell, an extension of Java language. The Arduino is the communication device between the computer program and the shutter that controls the timing. The program developed in several stages; in initial versions the camera was recording during the laser flashes (b). There are two versions of the final program. The first version takes an image before any bleaching occurs, turns the laser on while the camera is not recording, then turns the laser off and records (c). The user is able to control the amount of time the laser is on, the amount of time the laser is off, and the number of times this cycle repeats. The second version is essentially identical but pauses the program during the cycle to allow a fluorescing particle through the laser spot (d). The same parameters can still be controlled by the user with the addition of the length of the pause and the number of cycles carried out before and after the pause.



Results

Laser Gate in Action: Clear Tracks

The laser gate is able to produce much clearer tracks than before by reducing the amount of fluorescing protein particles. Some IFT tracks in this kymogram can be seen moving through the bleached area while some become bleached. Some IFT particles can also be seen staying stationary at the tip while they are remodeled. The stationary portion of the track reflects the time required for the remodeling of the IFT particles at the tip.



Future Applications

Cargo unloading

Cilia grow by addition of subunits to the tip. The laser gate will allow imaging of single precursor proteins as they are released from the IFT carriers and added to the growing cilium.

IFT vs. diffusion

Proteins in cilia can be transported by diffusion in addition to IFT. The laser gate program could be used to determine if diffusion is a viable mode of transport to the tip by adjusting the timing of the laser flashes. The speed of IFT is about 2 μm/s while the speed of diffusion is roughly 5 – 15 μm/s . Therefore, the speed of the laser gate can be adjusted so that the slower IFT particles cannot make it through unbleached while a few of the faster diffusion particles may be able to escape the laser spot unbleached.

Summary & Conclusions

- TIRF microscopy and GFP are used to visualize and image IFT in *Chlamydomonas* cells.
- Micro Manager and an Arduino are used to develop a laser gate program.
- The laser gate program allows entrance of single fluorescent particles into the cilia, greatly enhancing imaging quality.

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