The original approach to calculating diffusion coefficients of a fluorescent probe from Fluorescence recovery after photobleaching (FRAP) is a classical biophysical method, that has been used extensively to study molecular diffusion, particularly in cell membranes. It consists of irreversibly photobleaching a fluorescently labeled diffusing species, and subsequently following the fluorescence recovery over time. Fluorescence recovery then can be used to characterize diffusion of bleached molecules out of, and unbleached molecules into the bleached area and so extract the diffusion coefficient (D) of the fluorescent species.

While the experimental setup though specialized is relatively straightforward, the interpretation of the results is complex, severely limiting the quantitative use of the technique. Originally, FRAP assumed photobleaching by a well-characterized Gaussian profile laser beam and measurement of fluorescence recovery with a strongly attenuated version of the very same Gaussian beam. This approach offers well-defined and relatively convenient initial and boundary conditions, allowing a closed-form solution of the diffusion equation [1]. The method, however, requires a very precise characterization of the bleaching laser beam, a technically challenging task.

With the introduction of digital imaging and the Laser Scanning Confocal Microscope (LSCM), a multitude of imaging variations of the technique became possible. For example:

1. bleaching a circular region using a Gaussian laser beam, while monitoring the fluorescence recovery of the entire cell using wide field illumination and a fast CCD camera.
2. bleaching a rectangular area with a scanning (Gaussian) laser beam and monitoring the recovery with a scanning beam, that images an area much larger than the bleached area (typical LSCM FRAP experiment)
3. bleaching a (rectangular) shape as above with a scanning (Gaussian) beam and monitoring recovery of the whole field of view using a spinning disk confocal microscope (CCD camera)

Importantly, all these approaches can be implemented on commercially available microscopes, making the FRAP technique accessible to cell biologists as well as biophysicists. Indeed, commercially available confocal laser scanning microscopes (CLSM) have standard, push-button, FRAP abilities, thus a recovery curve can be easily extracted from the stack of images, following bleaching of any shape for any duration and with any intensity. In this realm, the shape of recovery curves from the very same sample can be very different, for different shapes of bleached area and different bleaching durations. These unknown, uncharacterized and uncontrolled initial and boundary conditions are often too complex to allow a precise closed, analytical solution of the diffusion problem [2]; a closed form solution does not exist even for a rectangular bleaching area, and an empirical one for calculating diffusion coefficients from a rectangular bleaching geometry, appropriate for imaging-FRAP in a confocal microscope, was later published [3]. This approximate solution allows quantitative FRAP for rectangular shapes of bleached areas. However, in commercially available equipment it is easily possible to choose initial conditions (e.g., size of bleached area, time of bleaching) which violate the assumptions required for even approximate solutions, and thus obtain calculated values of D that differ by orders of magnitude from the actual molecular diffusion coefficient. All these problems limit in fact the range of quantitative FRAP applications.
We have developed an alternative approach [4] to extracting diffusion coefficients directly from FRAP image stacks. Rather than approximate diffusional recovery from the data for a limited set of initial conditions, we run a 2D stochastic simulation of recovery and fit it to the observed data. We are using simulation in order to predict the recovery over time of an experimentally acquired bleached region, and then, comparing this prediction with the real (experimentally acquired) recovery, we extract the absolute diffusion coefficient that leads to best fit of the simulated recovery to the real one.

![Figure 1. FRAP recovery series (b-d) and its corresponding simulated recovery (e-g).](image)

References: