ADDITIONAL INFORMATION

For more about *DeltaVision*, see the following:

- *Online Help* provides reference information and procedures for using softWoRx.
- The *SoftWoRx Imaging Workstation User's Manual* shows how to process, visualize, and analyze data.
- The *DeltaVision Spectris System Manual* shows how to acquire data and how to maintain the data acquisition system.
- *Getting Started with QLM* Shows how to acquire photokinetic data.

REFERENCES

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Product Note

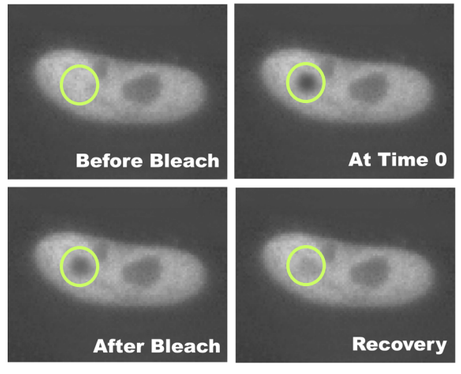
DeltaVision

Fluorescence Recovery After Photo Bleaching

INTRODUCTION

The Fluorescence Recovery After Photo-bleaching (FRAP) experiment method consists of photo bleaching a point (or points) of interest and then observing the recovery of fluorescence in the bleached area.

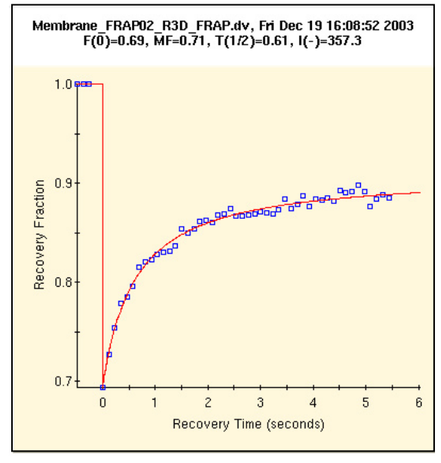
The recovery of fluorescence in the bleached area is measured as a function of time. From these measurements, you can infer information about the environment of the fluorescent molecules and their affinities.



A point of interest (indicated by the green circle) is photo bleached and monitored

The observation of recovery indicates that the fluorescent molecules are mobile. The rate of recovery is an indication of the speed at which the molecules are moving.

When you use the *DeltaVision* Quantifiable Laser Module (QLM) to perform FRAP, the point of interest is photo-bleached with a laser pulse and time-lapse images are captured to monitor the bleached area.



DeltaVision provides quantitative FRAP data

FRAP involves biology, chemistry, and physics and is a powerful method for measuring the dynamics of biomolecules. It provides quantitative data that can be useful for a number of types of biological studies, including:

- Affinity
- Biomolecular Cycling
- Biomolecular Environments
- Structural Kinetics

BACKGROUND

FRAP was first demonstrated by Poo and Cone in 1973. In 1976, Axelrod presented mathematics that enabled quantitive FRAP from focused laser spots on two-dimensional structures. The technique was popularized in general membranes by Jacobson (1976+), Golan (1980+), and many others. It fell out of popularity, based on work by Vigers, *et al.* (1988).

FRAP was resurrected by Cole, *et al.* in 1996 with the first use of GFP-chimera to study Golgi membrane mobility. Later in 1996, Lippincott-Schwartz popularized FRAP and other applications using GFP. Since that time, FRAP has gradually regained acceptance as a method for studying biomolecular dynamics.

The *DeltaVision* QLM option enables a new level of FRAP quantitation and discovery.

SINGLE AND MULTIPOINT FRAP

You can use the *DeltaVision* QLM module to monitor one or more points of interest in a specimen.

- Use Single-Point FRAP experiments to monitor a single location or to monitor several locations in a sequential fashion.
- Use Multi-Point FRAP experiments to monitor several locations in the sample at the same time.

You will typically run Single-Point FRAP experiments as a starting point for a research project. After you run Single-Point experiments and gain an understanding of the mobility parameters, you may decide to use Multi-Point FRAP.

Single-Point FRAP

Perform Single-Point FRAP experiments to investigate the mobility parameters of the fluorescent biomolocules at a single location within the specimen.

You can perform consecutive Single-Point FRAP experiments to monitor different locations in a sample.

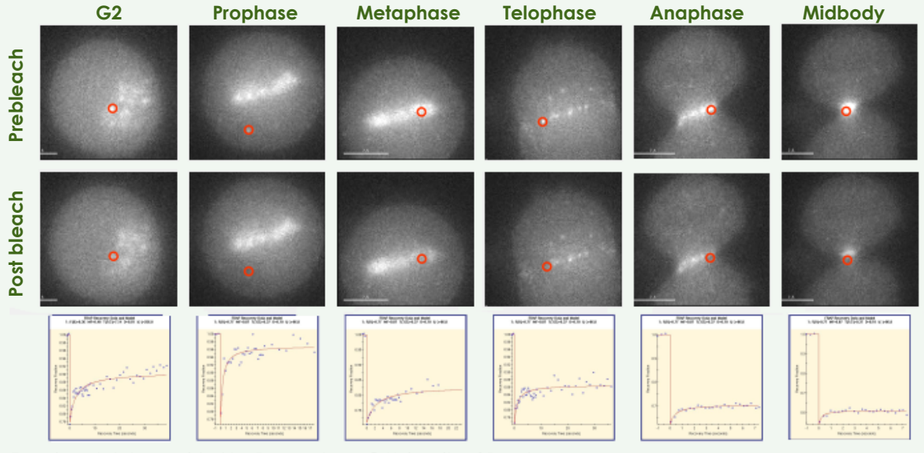
Single-Point FRAP experiments are the best way to measure fast recovery times.

Multi-Point FRAP Experiments

Multi-Point FRAP experiments allow you to investigate several points in a specimen at the same time. This is useful when the recovery rate is slow compared to data acquisition rate. It is also a convenient way to collect control data at the same time as you collect experimental data.

Monitoring Cell Division with Single-Point FRAP

These Single–Point Experiments monitored protein bound to kinetichores at different points of the cell cycle from G2 through Anaphase. Over 20 Single-Point FRAP experiments were performed on the same cell throughout cell division (six of these are shown). This cell survived and remained viable after the experiments.



Experiment data provided by P.A. Andrews @ University of Dundee

Evaluating and Reporting Results

Biomolecular mobility results often contain a lot of statistical variability. You will typically need to average multiple FRAP experiments. CV values of 50% or more are common. Many researchers feel that at least eight experiments are required to obtain meaningful averages and standard deviations.

The most commonly published FRAP results are Mobile Fraction and Half-Time or Mobile Fraction and Diffusion Coefficient. Often, a representative graph is shown along with a table containing the averages and corresponding standard deviations. The following table displays the statistically combined results from the four FRAP experiments shown below.

Experimental Condition	Mobile Fraction	Half-Time (secs)	Samples
Abnormal Cells	0.81 ± 0.06	1.76 ± 0.05	4

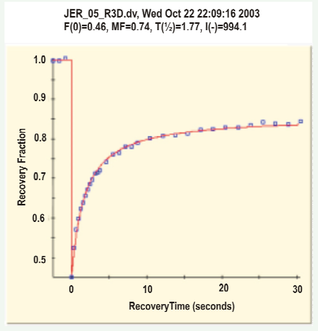
These data were measured under extremely stable conditions and are therefore very repeatable.

If you measured the beam radius and you feel confident that the 2D diffusion recovery model represents what is happening with your biology, then the 2D Diffusion Coefficient can be reported instead of the Half-Time, as shown below:

Experimental Condition	Mobile Fraction	Diffusion Coeficient (μm²/sec)	Samples
Abnormal Cells	0.81 ± 0.06	0.142 ± 0.004	4

To be thorough, the table should also have control results from normal cells. In addition, the table should report the statistical significance of the difference between the normal and abnormal results.

For many types of experiments, a Student's t-test is a good way to calculate the probability (*p*) of the null hypothesis. (That is, the probability that the two means are actually the same.) Smaller *p* values correspond to more significant differences. Example results are shown below.

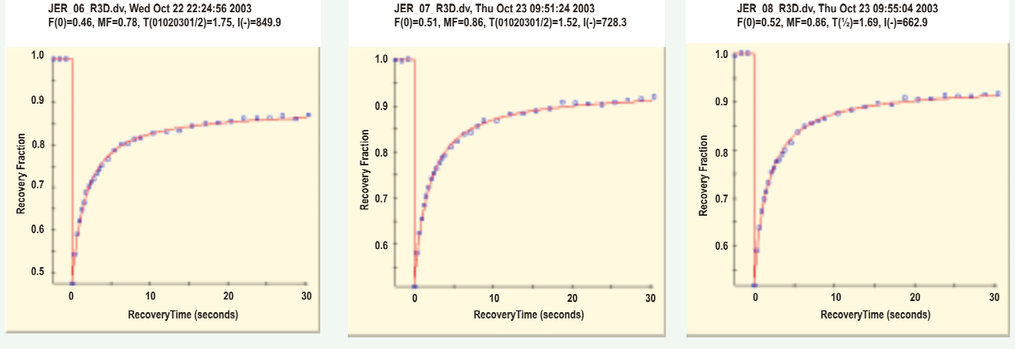


Experimental Condition	Mobile Fraction	Diffusion Coeficient (μm²/sec)	Samples
Abnormal Cells	0.81 ± 0.06	0.142 ± 0.004	4
Normal Cells*	0.52 ± 0.08	0.102 ± 0.011	4
Probablity *(p)*	< 0.002	< 0.003	4

*The results for normal cells are for example only and are not from real data.

Repeating FRAP Experiments

These four graphs were acquired as part of a small mobility study.

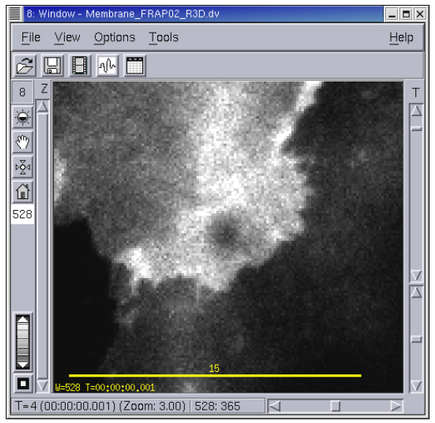


The FRAP Results Image

The FRAP Results Image is automatically displayed in the output window that is specified in the FRAP Data Analysis dialog. This image file includes the following three channels:

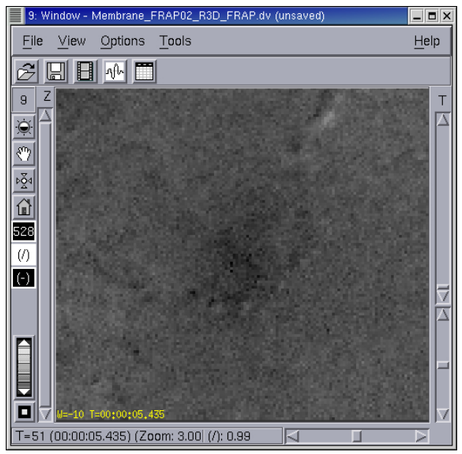
- The first channel is processed image data.
- The second channel, (/), is the FRAP Ratio Image.
- The third channel, (-), is the Spot Region of Interest Image.

The Processed Image data channel shows the original fluorescence image data. It includes the normalizations and corrections that you specified in the FRAP Data analysis dialog.



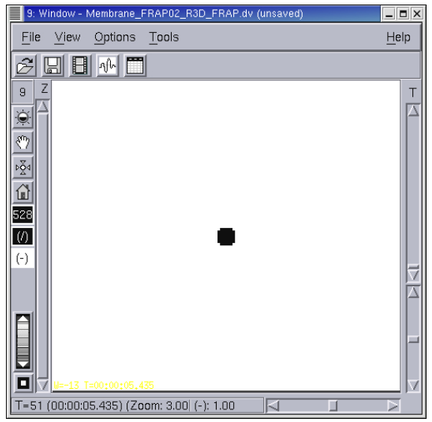
The Processed image data channel

The FRAP Ratio image displays the ratio of each time point divided by the average of the pre-bleach frames. This ratio demonstrates changes that occurred during the course of the experiment.



The FRAP Ratio image Channel

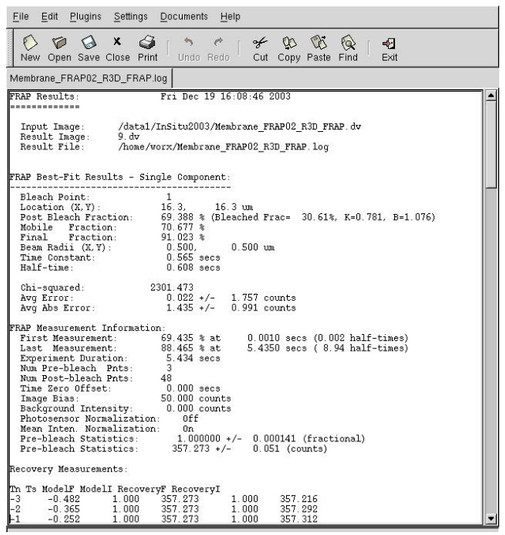
The Region of Interest image shows the location of the region of interest and the data values used for the analysis.



The Region of Interest Image channel

The Log Analysis File

The log analysis file contains a record of the FRAP analysis.

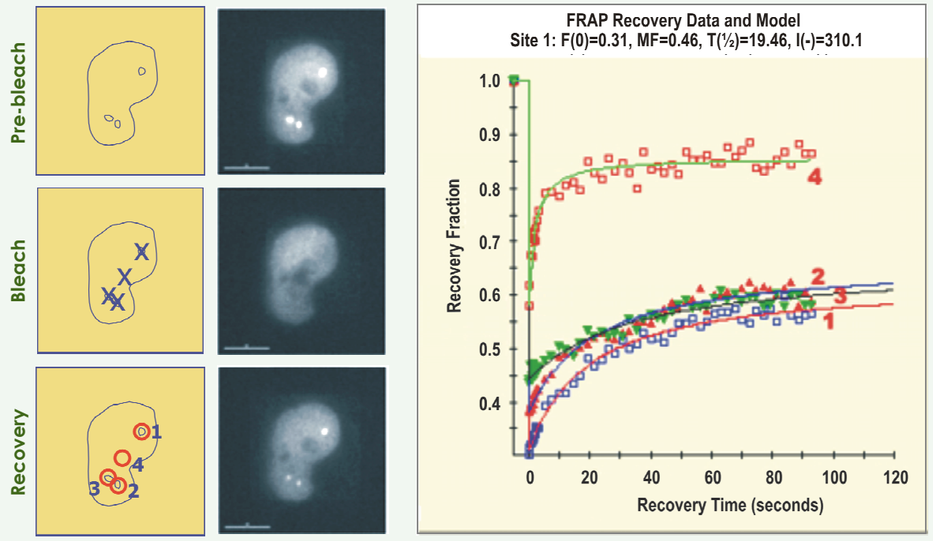


This log includes:

- Experiment log information
- Analysis details
- Tables for spreadsheet programs
- Experiment and model data

Monitoring Intracellular Differences With Multi-Point FRAP

This Multi–Point Experiment monitored three small structures in an area of the nucleoplasm. A fourth point in the nucleoplasm was used as a control point. The Fluorescence Recovery plot shows that the fluorescent probe within all three structures is behaving similarly and is not the same as the nucleoplasm.



Experiment data provided by P.A. Andrews @ University of Dundee

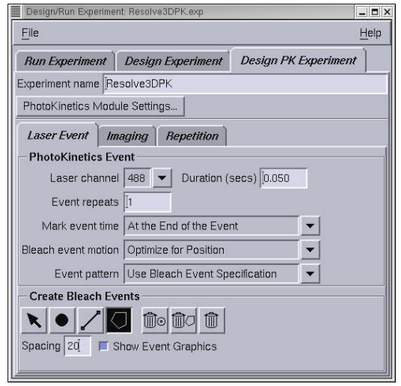
PERFORMING FRAP EXPERIMENTS

The procedure for performing Single-Point experiments is very similar to the procedure for Multi-Point experiments. Use the following instructions along with those for configuring and aligning lasers in the *QLM Getting Started Guide*.

►To perform a FRAP experiment

1. Configure and align the lasers as shown in the QLM Getting Started Guide.

2. From the main Resolve3D window, click **Experiment** to open the Experiment Designer. Then click the Design PK Experiment tab.



The Design PK Experiment tab settings define the nature of the photokinetic event, the timing of the event, and the imaging conditions to associate with the event. They also define the locations of the events.

3. In the **Experiment Name** field, enter the name of the macro.

Note *SoftWoRx* overwrites the default *Resolve3DPK* macro name without warning. If you select another name, *softWoRx* displays a warning message before it overwrites a preexisting macro.

4. Click the Laser Event tab. In the **Laser Channel** list, select which laser to use in the experiment.

5. To change the length of time (in seconds) that the laser shutter is open, enter a time in the **Duration** field.

6. To display or change laser power, click **PhotoKinetics Module Settings** and change the value of the **Power** field for the laser that you are using.

Tips
#1 For most FRAP experiments, short laser pulses provide better results than long pulses. Increasing the laser power can enable shorter bleach durations. When working with sensitive samples, however, you may need to reduce the power.

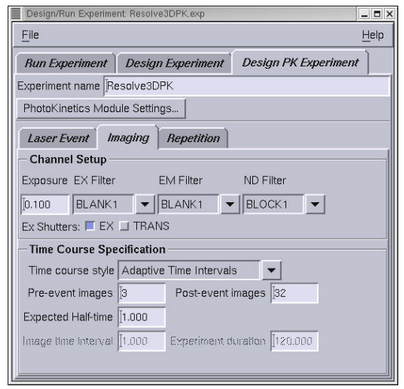
#2 *DeltaVision* prevents light from reaching the eyepieces when the laser is on but you can still view the sample through the camera.

7. In the **Mark event time** list, select the type of zero time ("t0") for the event. (You can specify the zero time at the start, middle, or end of the laser event.) In most cases, the best choice is the end of the event.

8. In the **Bleach event motion** field, select whether to optimize for speed or for position accuracy. (When you select to optimize for speed, minor stage position corrections are not performed.)

9. In the **Event pattern** list, choose one of the following options.
   - To perform a single-point FRAP experiment, choose **Use the Current Center Point**.
   - To perform a multi-point FRAP experiment, choose **Use Bleach Event Specification**.

10. Click the Imaging tab and select the exposure time, filters, and Time Course settings to use for data collection throughout the acquisition phase of the experiment.



11. Choose **File** | **Save As** to open the File Save As dialog and save the file.

Note The file size is calculated and reported in the Estimated File Size field, based on the Image Size selected in Resolve3D. If you change the image size, the Estimated File Size feature estimates the actual file size incorrectly unless you recreate the macro.

12. Click the Run Experiment tab and select **Start Scan**.

Note After you create and save an experiment macro, you can reload and run it later.

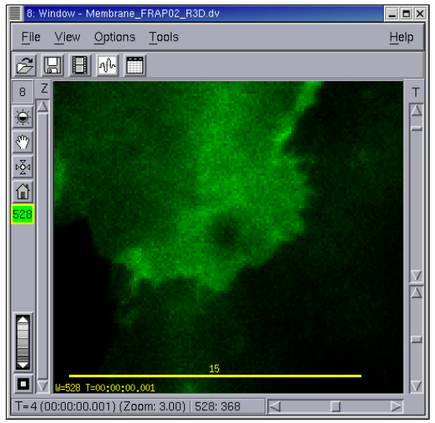
13. To run another Single-Point experiment on the same sample, center another point and click **Start Scan**. *DeltaVision* runs an experiment for the new point of interest.

ANALYZING FRAP DATA

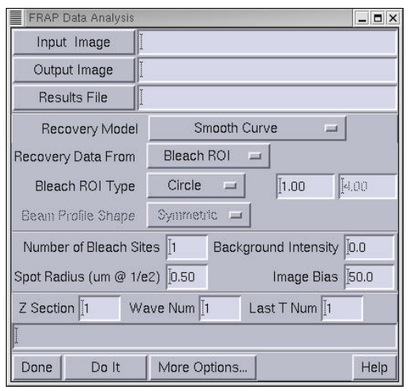
Use the following instructions to analyze FRAP data with the default FRAP Analysis settings.

►To analyze the FRAP data

1. Open the file that you want to analyze in an Image window.



2. From the main SoftWoRx window, choose **Measure** | **FRAP Analysis** to open the FRAP Analysis window.



3. Drag the window number of the image to the **Input** field or enter the file name in this field.

4. If you are using a Multi-Point FRAP data set, enter the number of bleach points in the **Number of Bleach Sites** field.

5. Click **Do it**.

Note To find out more about parameters such as recovery models, areas from which the FRAP data are obtained, background intensity and image bias, see the online Help.

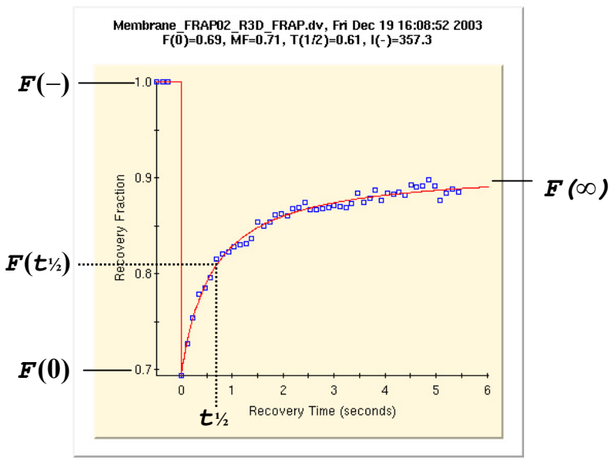
INTERPRETING FRAP DATA

The FRAP Analysis dialog provides three data files that you can use to interpret results:

- The Fluorescence Recovery Graph is a JPEG file that provides a time-point graph of the data.
- The FRAP Results image is a three channel image file that contains the original image, a ratio image, and a region of interest image.
- The Log Analysis is a text file that contains the data and analysis results.

The Fluorescence Recovery Graph

The Fluorescence Recovery graph is automatically displayed when the analysis is complete. This graph shows the recovery of fluorescence in the bleached area. The fluorescence recovery is expressed as a fraction of the fluorescence before the event. The Recovery Time starts at the bleach event. Data before the bleach event have negative time values.



The following basic FRAP values are used to calculate additional FRAP results.

Basic FRAP Values	Definition
`F(-)`, Initial Fraction	The initial fluorescence before the bleach event. When using ratio data, `F(-)` is nominally 1.
`F(0)`, PostBleach Fraction	The fraction of fluorescence that remains after the bleach event.
`F(∞)`, Final Fraction	The final fraction of fluorescence after an infinitely long recovery period.

The most informative FRAP results are described below:

Mobile Fraction (MF) is the fraction of fluorescent molecules that are free to move within the bleached region. It is calculated from:

$$MF = \frac{F(\infty) - F(0)}{F(-) - F(0)}$$

Half-time for Recovery ($t_{1/2}$) is the time required to recover half of the mobile fraction. That is, the time at which the fluorescence reaches ½MF.

$$\frac{1}{2} MF = \frac{F(t_{1/2}) - F(0)}{F(-) - F(0)}$$

The half-time is calculated from Axelrod's time constant and bleach correction factor, both of which are solved during the FRAP data curve-fit.

Diffusion coefficient (D) represents the rate (in μm^2/sec) of two-dimensional diffusion of the fluorescent molecules (see Axelrod, 1976). *D* is calculated from:

$$D = \frac{w^2}{4t_D}$$

where *w* is the laser spot radius and t_D is Axelrod's time constant.

The calculation of D is only valid when the fluorescence recovery arises from 2D diffusion and when the spot radius (*w*) is known. From the equation above, it is evident that errors in the measurement or estimation of *w* will propagate in a squared fashion to the calculated diffusion coefficient.