Get Ready to Crack CSIR NET 2021 [Short notes on Fluorescence Recovery After Photobleaching (FRAP)]

FRAP

(Fluorescence Recovery After Photobleaching)

FRAP (Fluorescence Recovery After Photobleaching) is used to measure the dynamics of two or three-dimensional movement of fluorescently labeled molecules within or between cells. The prime objective of FRAP is to assess molecular mobility which is an important parameter in the understanding of cell physiology.
The technique was developed by Axelrod and coworkers as a method to study protein mobility in the membrane of living cells.

PRINCIPLE:

The principle of FRAP is to photo bleach the fluorescently labeled molecules in a small region of the sample. Then the mobility of the fluorescently labeled molecules is evaluated from recovery of fluorescence due to exchange of fluorescently labeled molecules from surrounding unbleached area. In FRAP experiments, the photobleached area is restricted and as the technique's name suggests, the recovery of fluorescence back into it, is monitored.

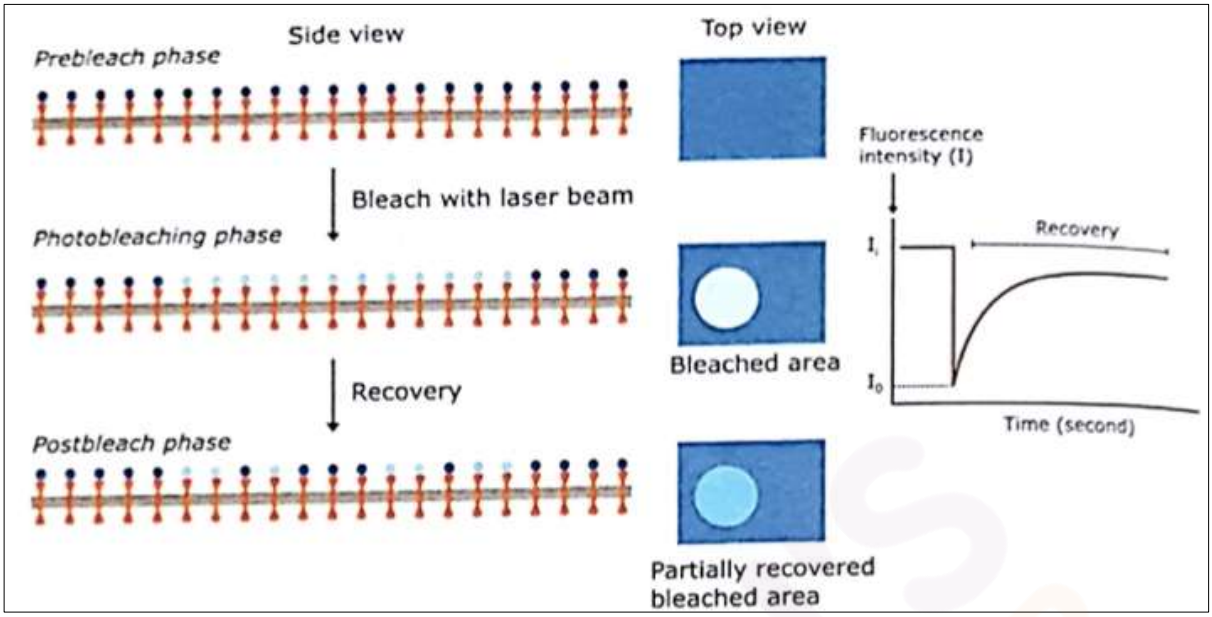
Photobleaching is an irreversible process that involves the irradiation of the fluorophore with light, resulting in the destruction of the fluorophore; and with it its ability to emit fluorescence. Recovery of the fluorescence signal is a result of the exchange of bleached fluorophores with those unbleached from the surrounding area. The fractions of molecules that are able and unable to participate in this exchange are termed the *mobile fraction* and *immobile fraction,* respectively.

PERFORMING FRAP:

The experimental setup comprises:

- a microscope (usually, confocal laser scanning microscopes)
- a light source and,
- a fluorescent probe coupled to the molecule of interest

Several images using a low light level are acquired to determine the initial fluorescence, and then a high level of light for a short time inside a region of interest is applied to bleach the fluorescence. Finally, another set of images using a light level sufficiently low to prevent further bleaching is acquired to gain insight into the redistribution of molecules via recovery of fluorescence.



A typical FRAP experimental setup. Here, the rate of lateral diffusion of a membrane protein is being measured.

⇒ This technique is composed of three phases- Prebleach Phase, Photobleaching Phase and Postbleach Phase.

⇒ A specific protein of interest is labeled with a fluorescent molecule (like GFP).

⇒ Fluorescent molecules are bleached in a small area using a laser beam. The fluorescence intensity recovers as the bleached molecules diffuse away and unbleached molecules diffuse into the irradiated area.

⇒ The diffusion coefficient is calculated from a graph of the rate of recovery: the *greater the diffusion coefficient* of the membrane protein, the *faster the recovery*.

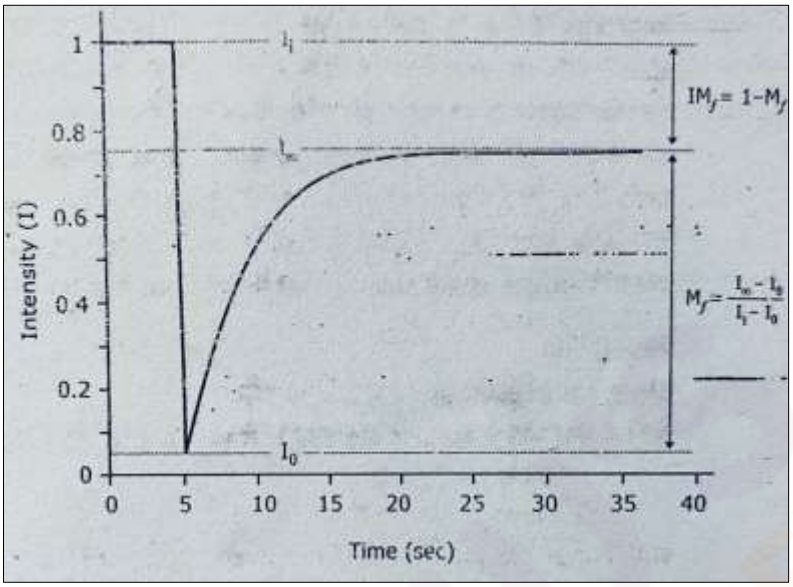
⇒ From this plot, the mobile and immobile fractions are determined by calculating the ratios of the final to the initial fluorescence intensity. In the graph,
Io is the fluorescence intensity immediately after the photobleaching,
$\mathbf{I_\infty}$ the fluorescence intensity after full recovery and,
$\mathbf{I_i}$, the initial fluorescence intensity before photobleaching.

Analysis of FRAP Curve:

- From the initial (prebleach) fluorescence intensity (I_i), the signal drops to a particular low value (I_0) as the high intensity laser beam bleaches fluorochromes in the region of interest. Over time, the signal recovers from the post-bleach intensity (I_0) to a maximal plateau value I_∞.

- From this graph plot, the mobile fraction (M_f) and immobile fraction (IM_f) is calculated.

- The information from the recovery curve (from I_0 to I_∞) can then be used to determine the diffusion constant and the binding dynamics of fluorescently labeled proteins.



A typical FRAP analysis curve

APPLICATIONS:

- Observing the rate of fluorescent recovery provides important insights into the movement and interaction of intracellular molecules.
- Analyse the mobility of individual lipid molecules within a cell membrane.
- Also study protein dynamics outside the membrane; a region of interest within the cytoplasm or even cellular structures within the cell.
- Apart from cell biology, FRAP has also been employed in the field of pharmacology:
  - FRAP has been used to non-destructively study the diffusion of therapeutic macromolecules in a variety of systems such as artificial gels, biological extracellular matrices and living cells.
  - Obtain information on the binding kinetics between therapeutic macromolecules and receptors inside living cells and on the flow rate of drugs in biological extracellular matrices.
- Although FRAP's advent in food science technology is not yet widespread, however, its ability to determine local diffusion properties in food samples with high precision makes it pretty useful tool for understanding the mechanisms controlling diffusion in foods.

- Diffusion influences the structure evolution during manufacturing, product shelf-life, the time-dependent development of the properties of the food during cooking, consumer preferences during consumption and the nutrient bioavailability.

FRAP VARIANTS:

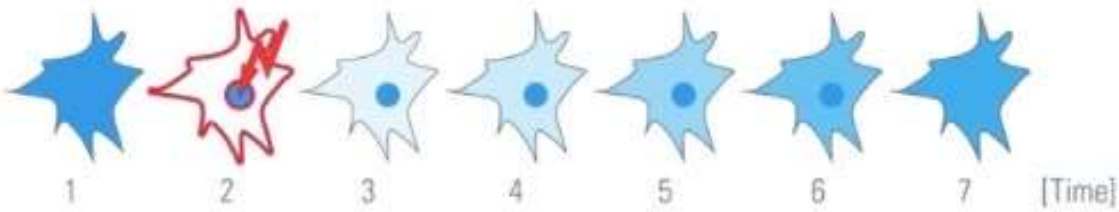
- Fluorescence loss in photobleaching (FLIP)

FLIP differs from FRAP by the repetitive bleaching of the same region in the specimen, thereof preventing recovery of fluorescence in that region. Here, a laser beam continuously irradiates a small area to bleach all the fluorescent molecules that diffuse into it, thereby gradually depleting the surrounding membrane of fluorescently labeled molecules.
FLIP is used to examine whether cellular organelles like ER or Golgi apparatus are interconnected. A FLIP experiment can predict whether molecules are mobile, immobile or restricted to compartments.

Starting at time point two, a region of interest (ROI) within a cell is bleached (arrow) repeatedly. Bleached molecules are spreading out. The loss of fluorescence indicates, whether cell organelles are physically connected.

- Inverse FRAP

In contrast to FRAP, inverse FRAP (i-FRAP) allows direct analysis of the fluorescent molecules. The fluorescent molecules outside an organelle are bleached. Thus, the efflux of fluorescent molecules out of organelles can be monitored directly without bleaching them. However, this method requires lot of light intensity to bleach the whole cell which poses a disadvantage.



At time point two, the whole cell area is bleached (arrow). Non bleached molecules are spreading out. The speed of fluorescence recovery in the cell can be measured.

- Fluorescence Localization After Photobleaching (FLAP)

Fluorescence Localization after Photobleaching (FLAP) is a ratio metric method which can be applied to two channels. It requires two different fluorescent labels that may be tagged to two proteins or one and only one of the two labels is bleached. The non-bleached population acts as the reference measure. The ratio of the bleached and of a second non-bleached area then gives insight into mobility of the proteins. In contrast to FRAP and FLIP, it is a direct measurement method and can be applied to structures which change their morphology rather fast.

At time point two, the ROI is bleached (arrow) with a suitable wavelength which only bleaches the red fluorescence,(as shown in the eg. above).

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