

## 5.5: Fluorescence on Membranes

The study of membranes poses a unique challenge in science due to their complex and fluid nature. Until recently, the majority of experiments was conducted in either fixed samples or on population levels that did not give insight into individual events. However, recent advances in fluorescence microscopy techniques have allowed scientists to visualize microscopic perturbations of individual vesicles and have allowed the study of membrane dynamics at higher spatiotemporal resolution than before.

### Direct Labeling of Lipids

The direct modification of lipids and membrane proteins has yielded several key discoveries in membrane biology, particularly in phase transitions. Fluorescent dyes are conjugated to either the polar head group or the hydrophobic tail. Fusion proteins consisting of a protein tethered to a fluorescent molecule such as GFP have also been used to study membrane protein interactions.

It was not until the 1990's that fluorescence experiments were used to directly probe lipid interactions. Fluorescent labels that bind to specific lipid phases were employed to show the effects of factors on phase separation in individual lipid vesicles. It was shown that factors such as pH, temperature, and cholesterol were all integral to ordered phase separation in lipid membranes [1,2,3]. Furthermore, by labeling different lipid types, it is possible to examine curvature of different phases within a single vesicle [4].

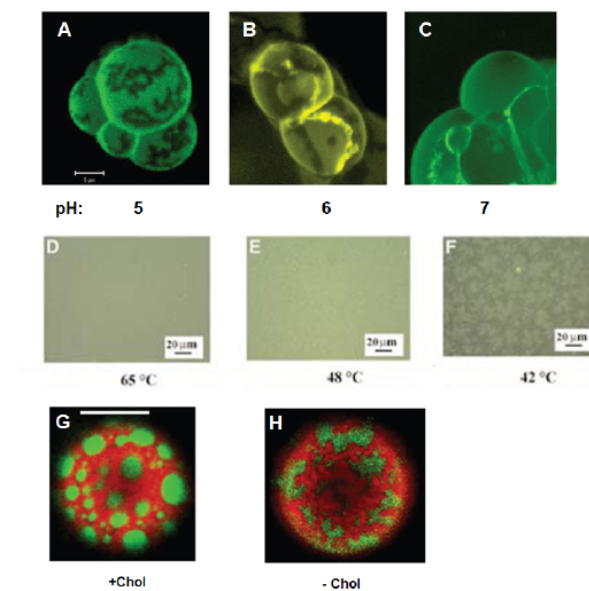


Figure 5.5.1: A,B,C) pH dependence of phase separation on giant unilamellar vesicles from human skin stratum corneal membranes. D,E,F) Temperature dependence on phase separation. G,H) Cholesterol dependence on phase separation on pig native lung surfactant.

### Total Internal Reflection Fluorescence Microscopy (TIRFM)

TIRFM uses an optical phenomenon that occurs at the transition between two mediums with different reflective indices. In the case of TIRF microscopy, the reflective indices are that of the glass coverslip and the sample (usually water based). If a beam of light is aimed at the critical angle, there is total internal reflection. When this happens, an evanescent wave is created that passes through the sample and decays exponentially. This results in the illumination of a region that is only ~100 nm in depth. The illumination of only the sample near the cover slip improves the signal to noise ratio at the membrane [5].

TIRF has been particularly important in the study of endocytosis at the membrane. A single endocytosis event occurs at a distinct puncta no larger than a few hundred nm in diameter. It also occurs over the course of seconds to minutes. TIRFM is ideal for studying these events in situ. It has allowed scientists to actively visualize the spatiotemporal properties of fluorescently labeled proteins for the purpose of developing selective inhibitors of dynamin facilitated endocytosis [6]. Irannejad et al effectively used TIRF to show that the receptors activate and continue to stay active during endocytosis and trafficking [7].

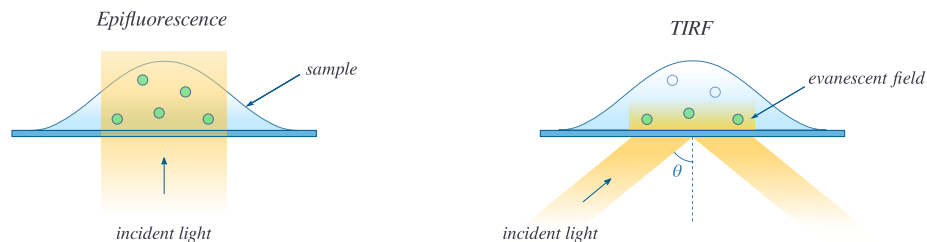


Figure 5.5.2: Schematic of Epifluorescent and TIRF-based imaging techniques. (CC BY-NC; Ümit Kaya)

## Super Resolution Microscopy

Previous fluorescence microscopy techniques are physically limited in the resolution that they can obtain due to Abbe's limit. Abbe's limit is determined by the medium and wavelength of light used. It dictates the maximum distance in which two distinct points of light can be visualized. The limit for modern spectroscopic tools is  $\sim 200\text{-}300\text{ nm}$ . However, microdomains on membranes such as lipid rafts are  $10\text{-}200\text{ nm}$  in width and therefore lie below the lower limit [12]. To circumvent this limitation, a number of techniques have been developed.

### STimulation Emission Depletion (STED) Microscopy

STED imaging is a confocal based technique that employs the sequential illumination of an area followed by the illumination of the surrounding area. The secondary illumination creates a red shifted emission that is not collected by the imaging device. The resulting illuminated area is orders of magnitude smaller than what is attainable with conventional microscopic methods. Furthermore, the rapid rate of data collection using STED has allowed the super resolution imaging of dynamic environments such as the membrane. In combination with single molecule tracking methods, scientists have been able to follow the movement of individual molecules across the membrane.

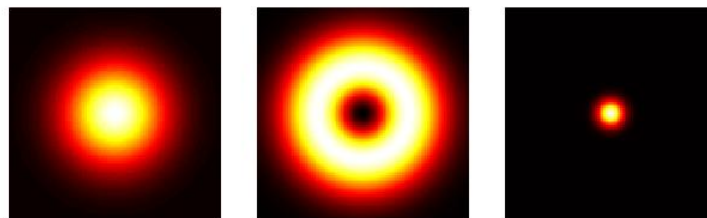


Figure 5.5.3: Illustration of STED Illumination. From left to right: the initial excitation, the secondary illumination, and the resulting illuminated area. Picture courtesy of Marcel Lauterbach

It has been shown by Mueller et al. that the diffusion of sphingolipids across a live membrane was much slower than that of other categories of lipids [8]. Sphingolipids are hypothesized to be enriched in lipid-raft environments. Furthermore, it was shown that the rate of diffusion increased when upon depletion of cholesterol or inhibition of actin polymerization. These discoveries furthered the development of the theory of both lipid rafts and the idea that the actin cytoskeleton and anchored membrane proteins serve as corrals that inhibit lipid diffusion [9].

### STochastic Optical Reconstruction Microscopy (STORM) and PhotoActivated Localization Microscopy (PALM)

Both STORM and PALM function on the same principle. When proximal fluorophores are simultaneously activated, their signals are impossible to dissociate. However, if they are stimulated and captured individually, they can later be resolved using computational methods. STORM uses antibodies labeled with pairs of inorganic dyes as activator/reporter dye pairs to create the stochastic activation of individual dyes. PALM employs photoswitchable or photoactivatable fluorophores to create stochastic activation and emission.

Recent developments in PALM have allowed the co-localization of Tetherin, a cytoskeleton anchoring protein, with that of lipid rafts. This gave evidence for the association of lipid rafts with cytoskeletal components for the effective separation exhibited in membranes. Furthermore, the synthesis of photoswitchable fluorophores that bind cholesterol and sphingolipids have allowed the direct visualization and measurements of cholesterol and sphingolipid enriched domains [10].

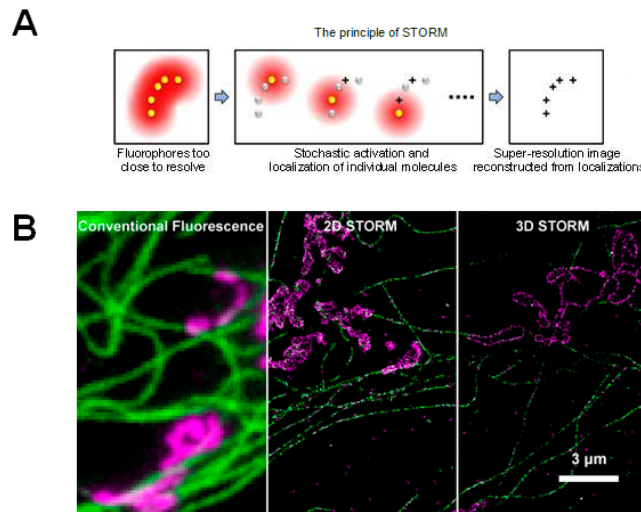


Figure 5.5.4. STORM super-resolution imaging. A) Schematic of STORM based techniques. B) Comparison of STORM to resolve mitochondria and microtubules near the membrane surface. (Figures courtesy of [bo.huang@ucsf.edu](mailto:bo.huang@ucsf.edu)).

### Fluorescence Recovery After Photobleaching (FRAP)

FRAP is an imaging technique that analyses the recovery of signal after a photobleaching event. In general, fluorescently labeled lipids or membrane proteins in a small area are exposed to extreme laser power. This causes irreversible destruction of fluorophores leading to a bleaching effect. Then a time lapse is used to determine the kinetics of recovery of signal in the area after photobleaching. The recovery time can then be used to determine the diffusion coefficient. The diffusion coefficient is directly proportional to the rate of diffusion.

$$D = \frac{w^2}{4t_D} \quad (5.5.1)$$

where  $D$  is the diffusion coefficient,  $w$  is the radius of the photobleached area, and  $t_D$  is the diffusion time.

The method has been extensively used to explore lateral diffusion of both lipids and proteins about various membranes [11].

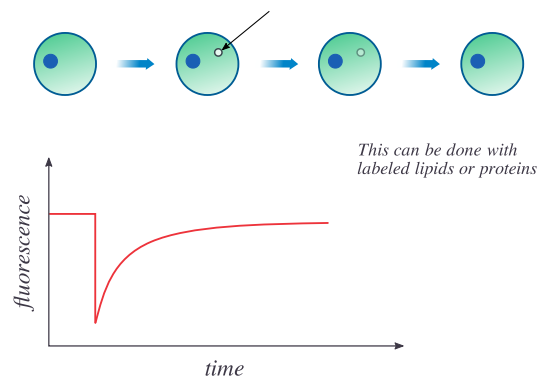


Figure 5.5.5. Diagram of FRAP. (CC BY-NC; Ümit Kaya)

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