TIRF – Spatial restriction reduces background noise

In superresolution microscopy methods, like PALM, individual fluorescent molecules such as tagged proteins are detected. In ordinary microscopes, this is rather difficult because light emitted or scattered by out-of-focus molecules confer a strong background to the image. Another source of unwanted fluorescent signal is the autofluorescence of some structures such as mitochondria, chloroplasts, or autofluorescing molecules such as NADH. All these sources of light tend to blur the fluorescence from the particular molecule of interest. Here, we will introduce a technique that allows the detection of single fluorophores without detection of the background noise: Total-internal reflection fluorescence (TIRF) microscopy.

When light travels from one material into another one, e.g., from air into water, it is refracted, i.e., the light rays do not have the same direction as they had before entering the second material. When light from a dense material with a high refraction index, e.g., glass ($n = 1.5$), travels into a less dense material, it is bent away from the normal of the surface (the normal is a line drawn perpendicular to the surface, shown as a dashed line in figure 1A). Figure 1B shows that if the angle between the incident ray and the normal is larger than a critical value, the light rays are bent such they do not leave the dense material, but travel at its surface. This angle is called the critical angle of total internal reflection; for light travelling from glass into air, this angle is $\theta_c = 42°$. At a larger angle, the light will be completely reflected back into the dense material.

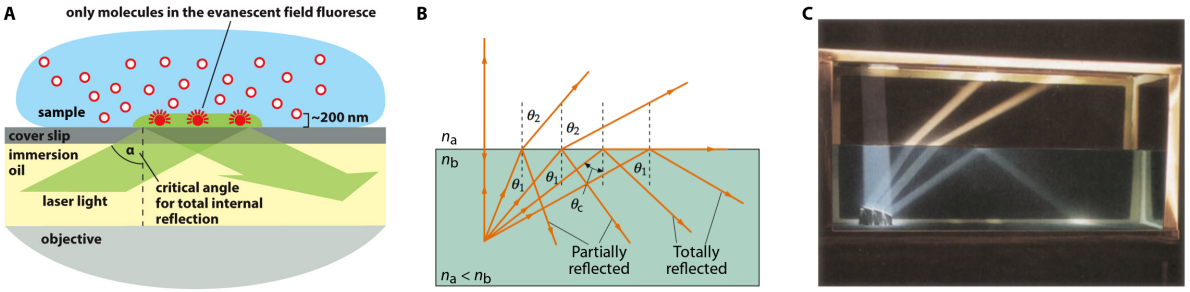

Figure 1 TIRF microscopy allows the detection of single fluorescent molecules. (A) TIRF microscopy uses excitatory laser light to illuminate the cover-slip surface at the critical angle at which all the light is reflected by the glass-water interface. Some electromagnetic energy extends a short distance across the interface as an evanescent wave that excites just those molecules that are attached to the cover slip or are very close to its surface. Note that TIRF microscopes often are inverted microscopes as shown here. In inverted microscopes, the objective is below the specimen pointing up. **(B)** Light travelling from a dense to a less dense material is bent away from the normal to the surface. If the angle of incidence θ is larger than a critical value, the light is totally reflected and does not leave the dense material. **(C)** Photograph of total reflection at an air-water surface. (A: From Molecular Biology of the Cell, Alberts *et al.*, 6th edition, Garland Science; B and C: From Physics, Tipler, 7th edition, SpringerSpektrum)

In a TIRF microscope, the specimen is illuminated with a laser at an angle where total internal reflection occurs. This is possible, because the refraction index of the glass cover slide is greater than that of water ($n = 1.3$). Because of total internal reflection, the light does not enter the sample, and the majority of fluorescent molecules are not illuminated. However, electromagnetic energy does extend, as a so-called evanescent field, for a very short distance (100 to 200 nm) beyond the surface of the cover slip and into the specimen, allowing just those molecules in the layer closest to the surface to become excited (see figure 1B). When these molecules fluoresce, their emitted light is no longer competing with out-of-focus light from the other molecules in the specimen, and can now be detected. TIRF has allowed several groundbreaking experiments, such as imaging of single motor proteins moving along microtubules.

What role does TIRF microscopy play in a PALM experiment? In order to minimize both autofluorescence and detector noise, images are taken in a TIRF-microscopy setup. The less noise from other sources is detected by the camera, the more reliably it can pick up the photons emitted by the molecules of interest. Without using a TIRF microscope, the positions of the individual fluorophores could not be determined at high-enough accuracy such that the advantages of the PALM method would be undone.