Two Light Steps for a Better View

The introduction of the green fluorescent protein (GFP) as optical marker in 1994 enabled a plethora of applications in biological and biomedical research, yielding revolutionary insight into various cell and protein dynamics [1]. Ever since, many proteins that cover the entire visible spectrum have been engineered and successfully used.

In addition, proteins were introduced that change the color reversibly or irreversibly upon light exposure of a particular wavelength. Two classes can be distinguished: i) photoactivatable and photoswitchable fluorescent proteins, respectively, that change from a non-fluorescent to a fluorescent state, and ii) photoconvertible fluorescent proteins that can change their fluorescent emission spectrum upon light-induced conversion [2,3]. The second class of proteins is of particular importance for biomedical imaging, as they can be visualized before and after photoconversion. This advantageous feature allows the experimenter to track both the non-photoconverted as well as the photoconverted protein population in parallel.

Photoswitchable Fluorescent Proteins

The green-to-red photoconvertible fluorescent proteins isolated from the Anthozoa group are the most widely used group of photoconvertible proteins. Kaede (Japanese for maple leaf) was the first protein that was isolated from the stone coral Trachyphyllia geoffroyi in 2002 [4]. Nowadays, a variety of green-to-red photoconvertible protein groups are in use with improved brightness and increased conversion efficiency. All proteins, from the Kikumen-GR-[5], ClavGR-[6], Dendra-[7], over the Eos-[8] to the Maple-protein group [9], change from green to red fluorescence upon violet light exposure.
The mechanism of photoconversion using violet light is identical for all protein groups: A C-C-bond cleavage results in the formation of a new C=C-double bond. This newly formed double bond increases the conjugated electron system, which eventually defines the spectral shift, resulting in the irreversible conversion from the green to the red fluorescent state [10].

Since their introduction more than 15 years ago, green-to-red photoconvertible fluorescent proteins are successfully employed as molecular markers in biomedical imaging.

Selective photoconversion of protein populations labeled with green-to-red convertible fluorescent proteins enabled the visualization of complex intracellular dynamics [11]. Cell organelles such as mitochondria could be investigated using photoconversion [12]. In addition, selected groups of cells could be followed during development in model organisms such as zebrafish [13] and mice [14]. Green-to-red photoconvertible fluorescent proteins have also been applied in various applications using super-resolution microscopy [15]. It is worth noting though that precise photoconversion is restricted to flat tissue and even cell culture, since violet light cannot be axially (i.e. in the z-direction) confined.

In 2015, my group developed an advanced modality of photoconversion [16]: the fluorescent protein Dendra2 could be converted from the green to red fluorescent state by the simultaneous delivery of blue (488 nm) and red to infra-red (730 nm) light. We referred to this 2-step mechanism of photoconversion as 'Primed Conversion'. During Primed Conversion, the green protein first absorbs the 488 nm ‘priming’ light, upon which it quickly enters a long-lived (i.e. a few milliseconds) intermediate state. Proteins in this so-called ‘primed state’ can then further absorb a far-red ‘converting’ photon, irreversibly converting them to red emitting fluorescent proteins. The red species created during this process is spectrally identical to the one created during the traditional photoconversion process using
violet light.

**Mechanism of Primed Conversion**

A series of isotope experiments allowed us to understand the underlying mechanism of Primed Conversion, and in particular the intermediate primed state [17]. We could show that this state is a case of a phenomenon known from quantum chemistry - a ‘triplet state’. After about five milliseconds, the fluorescent protein Dendra2 returns to its ground state. Primed Conversion happens only if the second phase - the illumination with red to near-infrared light - occurs within the triplet time window.

The duration of the triplet state depends greatly on the stability of the fluorescent protein. This, in turn, depends on the exact sequence of the amino acids around the chromophore (i.e. the protein’s color center). In a systematic amino acid-screen, we could show that the triplet state and hence Primed Conversion can be influenced by sequence position 69 [17]. The introduction of the polar amino acid Threonine at this position proved sufficient to recover Primed Conversion for virtually all fluorescent protein families that did not carry this mutation (i.e. the Eos family, KikGR, and Kaede).

The created “pr”- (for primed convertible) variants, the pr-Eos family, pr-KikG, and pr-Kaede, were not just made switchable in two phases for the first time; they are also more stable and therefore brighter. These features will render Primed Conversion highly attractive for biomedical imaging.

**Application in Microscopy**

We made the original discovery with a laser not conventionally available, using light in the near-infrared range (730 nm). Nowadays, we have demonstrated that the effect can also be achieved using conventional red lasers found in every commercial fluorescence microscope (around 640 nm) [17]. Hence, Primed Conversion can be performed with any fluorescence microscope without any extensive technical adjustments, making it available to a broad user base.

Primed conversion can be used in microscopy in order to mark a narrowly defined point in a tissue sample. We accomplished axially confined photoconversion by selective intersection of priming and converting beam in a common focal volume [16,18]. Primed Conversion occurs only at this intersection. Because neither blue nor red laser light have a toxic effect, the method is ideally suited for living organisms. By focusing the combined laser beam’s focal point, we could highlight a single neuron with all its extensions in a live, anesthetized zebrafish [16].
The introduction of novel, improved fluorescent proteins for Primed Conversion made applications with other microscopy techniques possible. In super-resolution microscopy, which has been around for several years now, primed convertible fluorescent proteins were utilized to render various structures of the cytoskeleton visible. For instance, we visualized different protein populations by combining Primed Conversion (i.e. blue and red light) of a pr-modified fluorescent protein, pr-mEos2, with a non-pr-modified protein, mEos2, using traditional photoconversion (i.e. violet light) [17].

Using the acquired knowledge, we are working together with protein experts to modify other fluorescent proteins used in microscopy in the same way. Recently, we modified in a collaboration a protein so that it can be split off from a gene-activating messenger in a way that allows it to be light-activated with two colors [19]. In the future, it will be possible to use axially confined Primed Conversion to activate specific genes in a single cell of the tissue. Moreover, photoconvertible proteins that detect calcium can be modified in this way as well and could be used with Primed Conversion [17]. Using modified reporter proteins, functional analysis using Primed Conversion could potentially benefit 3D brain activity mapping.

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References