

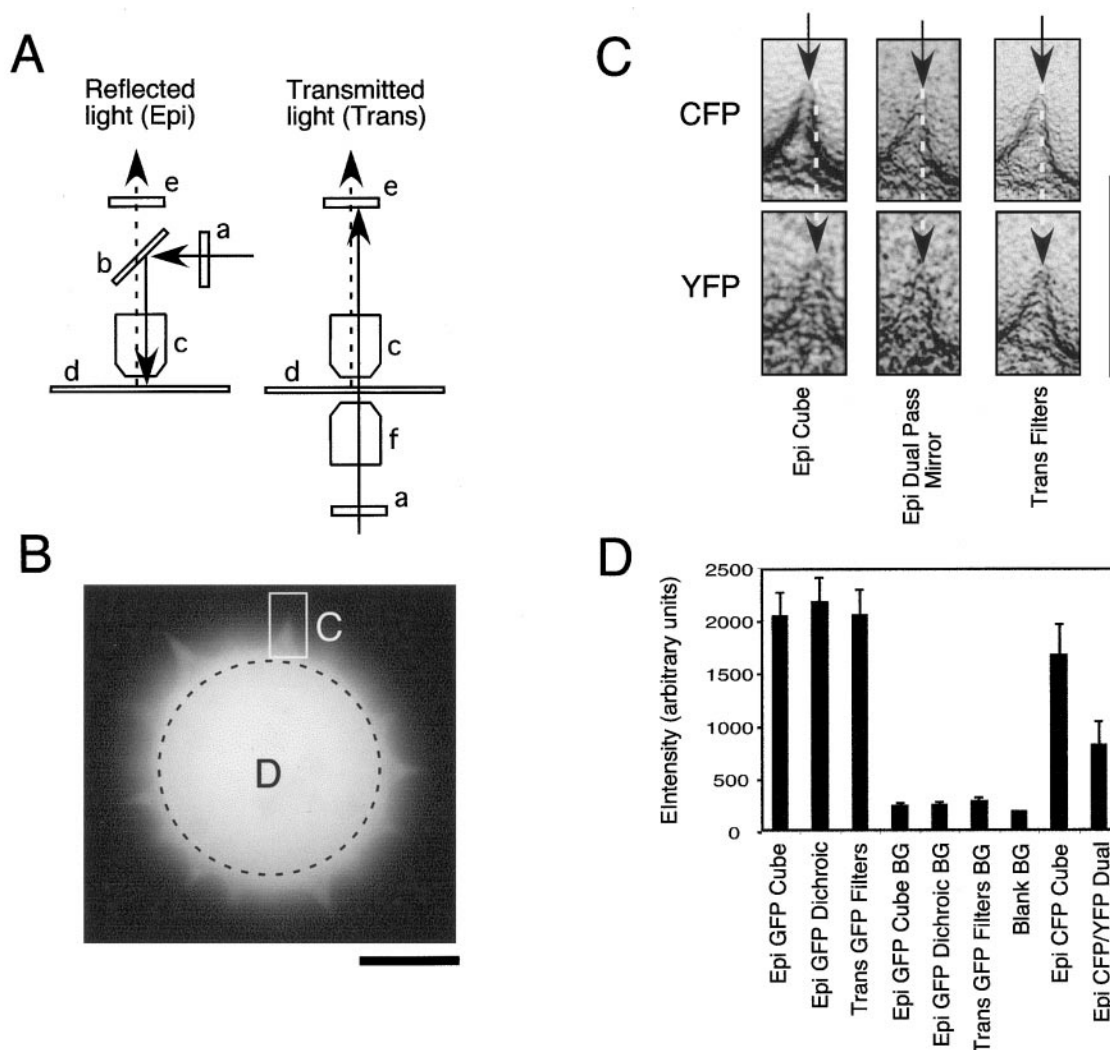
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### Transmitted Light Fluorescence Microscopy Revisited

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From its introduction in 1967 by Ploem (1), reflected light fluorescence microscopy, commonly called “epi-fluorescence,” has enjoyed wide acceptance. Its optical path is relatively simple: full-spectrum light passing through an excitation filter is reflected by the dichromatic mirror into the objective lens to illuminate the sample; the excited sample emits fluorescent light, which is re-collected by the objective lens and passed through the emission filter to the camera.

The recent development of biosensors based on genetically encoded variants of green fluorescent protein (GFP), coupled with advances in digital, multi-modes, epi-fluorescence microscopy, has introduced new powerful tools for observing protein dynamics and protein-protein interactions at high spatial and temporal resolution within living cells. However, there are some disadvantages inherent in epi-fluorescence microscopy: a) mechanical switching of filter cubes to



**Figure 1.** Intensity and image misalignment comparisons between reflected light fluorescence microscopy (epi) and transmitted light fluorescence microscopy (trans). (A) Schematic diagrams of the optical paths of epi- and trans-fluorescence: a-excitation filter, b-dichromatic mirror, c-objective lens, d-specimen, e-emission filter, f-condenser lens. (B) Fluorescent image of a pollen grain. The rectangle C highlights a pollen spike that is used to measure image misalignment. The dashed circle D represents the area of the pollen grain used to measure fluorescent intensity. (C) Positions of a pollen spike (edge-enhanced) imaged with epi- and trans-fluorescence. (D) Mean fluorescence intensities of pollen grain measured with epi- and trans-fluorescence. See text for further details of microscopy set-up. Bar = 10  $\mu$ m.

view different color fluorescence can cause misalignment of images; b) multi-pass filter cubes can eliminate the misalignment problem, but may attenuate the emission light; and c) the epi-fluorescent light source cannot be used in combination with transmitted light techniques such as Phase or differential interference contrast (DIC) microscopy.

We investigated the feasibility of using transmitted light fluorescence microscopy (referred to here as "trans-fluorescence") to overcome the limitations of epi-fluorescence described above. In trans-fluorescence, the specimen is excited by light passing through the condenser lens, and the fluorescent emission is captured by the objective lens. This mode eliminates the need for dichromatic mirrors. Trans-fluorescence was pioneered prior to epi-fluorescence but was largely abandoned due to two major drawbacks. In the past, excitation light coming from the condenser lens and going through the objective lens could not be completely blocked by the emission filter, leading to a high background signal. Furthermore, optical alignment and matching of the condenser lens and the objective lens were often difficult, leading to non-optimal fluorescence. However, recent developments in filter technology and automated microscope controls prompted us to test whether trans-fluorescence could provide significant improvement over epi-fluorescence in alignment and fluorescent intensity.

Figure 1A shows schematic diagrams for the epi- and trans-fluorescence optical set-ups. We used a Nikon Eclipse 800 upright microscope with the following attachments. For epi-fluorescence, a first excitation filter wheel (Sutter Instruments, model Lambda 10-2) equipped with band pass excitation filters CFP (436/10 nm), GFP (HQ 525/50 nm), and YFP (535/30 nm) (Chroma Technology) was placed at the epi portal; a corresponding dichromatic mirror for each color protein was placed in the filter cube holder, a Plan Apo 100 $\times$ /1.4 N.A. oil objective lens was used; and a second filter wheel equipped with band pass emission filters CFP (470/30 nm), GFP (HQ 470/40 nm), and YFP (500/20 nm) was placed at the camera port. For trans-fluorescence, minor modifications from the above set-up were required. The excitation filter wheel with appropriate filters was placed at the trans portal; a Universal 1.4 N.A. oil condenser lens was used to illuminate the sample; and all dichromatic mirrors were removed from the optical path. The same 100-watt mercury arc lamp light source was used for illumination in the epi and trans portal. Images of a pollen grain (Carolina Biological) were captured by an Orca-100 cooled CCD digital camera (Hamamatsu Photonics) controlled by the software package OpenLab 3.0 (Improvision). Images were captured at an exposure time of 100 ms.

To compare differences in signal intensity between epi- and trans-fluorescence, we imaged a pollen grain with the GFP filter set using

the two modes. Figure 1B shows the image of the measured pollen grain. The average intensity values of the pollen grain (over area "D"), in 5 separate measurements, were:  $2056 \pm 224$  pollen/ $252 \pm 23$  background for the Epi GFP filter cube,  $2190 \pm 221$  pollen/ $257 \pm 25$  background for the Epi GFP filter wheels/dichromatic mirror set-up, and  $2062 \pm 240$  pollen/ $292 \pm 39$  background for the Trans GFP filter set-up (Fig. 1D). We conclude that the fluorescent signal intensities obtained with the epi or trans methods are not different, although the trans method produced slightly higher background intensity. The trans-fluorescent technique is a comparable alternative to epi-fluorescence.

To compare image alignment between epi-fluorescence and trans-fluorescence, we imaged pollen grains sequentially with the CFP and YFP filter sets, using the epi mode and then using the trans mode. Switching between CFP and YFP filter cubes and dichromatic mirrors was done manually, while switching between CFP and YFP filters was automated *via* the filter wheels. Use of the epi mode with manual switches produced a significant lateral shift of up to 1  $\mu$ m between the CFP and YFP images (Fig. 1C). The epi mode using automated filter switches with CFP-YFP dual-pass mirror and the trans mode using automated filter switches alone produced no measurable image shift (Fig. 1C). However, the epi mode with CFP-YFP dual-pass mirror attenuated the fluorescent light by 50% compared to the epi mode using filter cube and the trans mode (Fig. 1D). We conclude that the trans mode produces better image alignment than modes using the manual switching of filter cubes; it also produces higher fluorescent intensity compared to the epi mode with a dual-pass mirror.

These studies suggest that transmitted light fluorescence microscopy may be an attractive alternative to reflected light fluorescence microscopy. The advantages of trans-fluorescence include: a) image alignment is better than modes using mechanical switching of filter cubes; b) image fluorescence intensity is comparable to the best epi set-up and is twice as bright as an epi mode using a dual-pass mirror and filter wheels; and c) fluorescence can be combined with Phase or DIC techniques using the same light source.

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### Literature Cited

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### The Process of Reducing CA1 Long-Term Potentiation by the Integrin Peptide, GRGDSP, Occurs Within the First Few Minutes Following Theta-Burst Stimulation

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Theta-burst stimulation (TBS) induces Schaffer collateral-CA1 synaptic long-term potentiation (LTP; 1, 2), an experimental model of synaptic plasticity believed to reflect physiological pro-

cesses during normal learning and memory. Various adhesion receptors may play a role in LTP (3), including integrins, transmembrane signaling receptors that link extracellular ligands to the