Optimizing Fluorescence Microscopy: Exploiting the benefits of Broadly Tunable Optical Parametric Oscillators

Fluorescence microscopy has revolutionized microbiology research by providing a non-destructive method for high-resolution imaging of living cells. This technique allows researchers to visualize the spatial distribution of molecules within cells by capturing the light emitted as fluorescence from optically excited molecules. It is invaluable for studying molecular interactions, structural organization, and dynamic cellular processes.

Not all molecules naturally fluoresce, so fluorescent labels, known as fluorophores, are often used to visualize them. Each fluorophore has a distinct excitation spectral band, which requires an illumination source that emits light at the corresponding wavelength for proper excitation. A significant challenge in fluorescence microscopy is the overlap of excitation bands when using multiple fluorophores, which can blur the imaging of different labels. Traditional illumination sources, like fixed-wavelength lasers or LEDs, lack the ability to precisely tune to the specific excitation bands of each fluorophore, limiting their usability and reducing image quality. To optimize fluorescence imaging, selecting the right excitation wavelengths for each fluorophore is crucial for minimizing interference and achieving high-resolution images. Many microscopy systems rely on fixed-wavelength lasers for multi-color imaging, but this approach restricts flexibility. Tunable lasers, such as optical parametric oscillators (OPOs), address this issue by enabling precise excitation of fluorophores across a wide wavelength range, covering ultraviolet (UV), visible (Vis), and infrared (IR) regions. This broader tunability enhances fluorophore compatibility and improves overall image resolution.

The **Oria IR femtosecond Optical Parametric Oscillator** **(OPO)** laser coupled with Oria VIS and Oria Blue second harmonic generators (and a Ti:Sapphire pump laser), which can be seen in Figure 1, is an ideal illumination source for performing different fluorescence microscopy techniques, such as linear fluorescence microscopy, two-photon microscopy or Fluorescence Lifetime Imaging Microscopy (FLIM).

Oria IR XT with Harmonic generators (SHG)

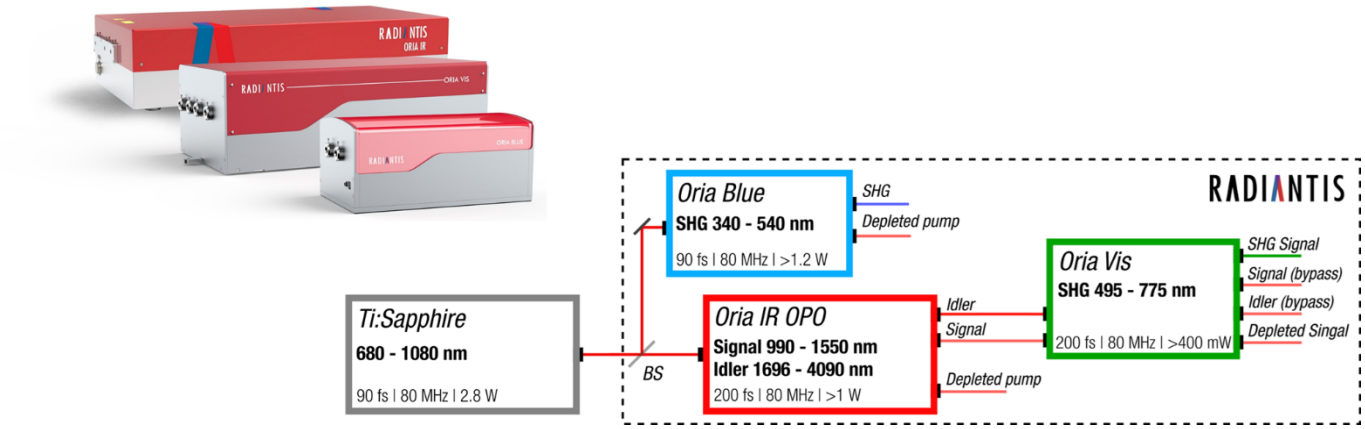

Figure 1: Scheme of Oria VIS and Oria Blue extension modules in combination with Oria IR OPO and Ti:Sapphire pump laser

The following text refers to linear fluorescence microscopy. The experimental set-up is represented in Figure 2. Coupled with a simple microscopy setup, we utilize the ORIA IR femtosecond OPO laser with ORIA VIS and ORIA Blue second-harmonic generation modules, obtaining a broadly tunable laser spanning 345 nm – 4090 nm, for analyzing biological samples through fluorescence imaging.

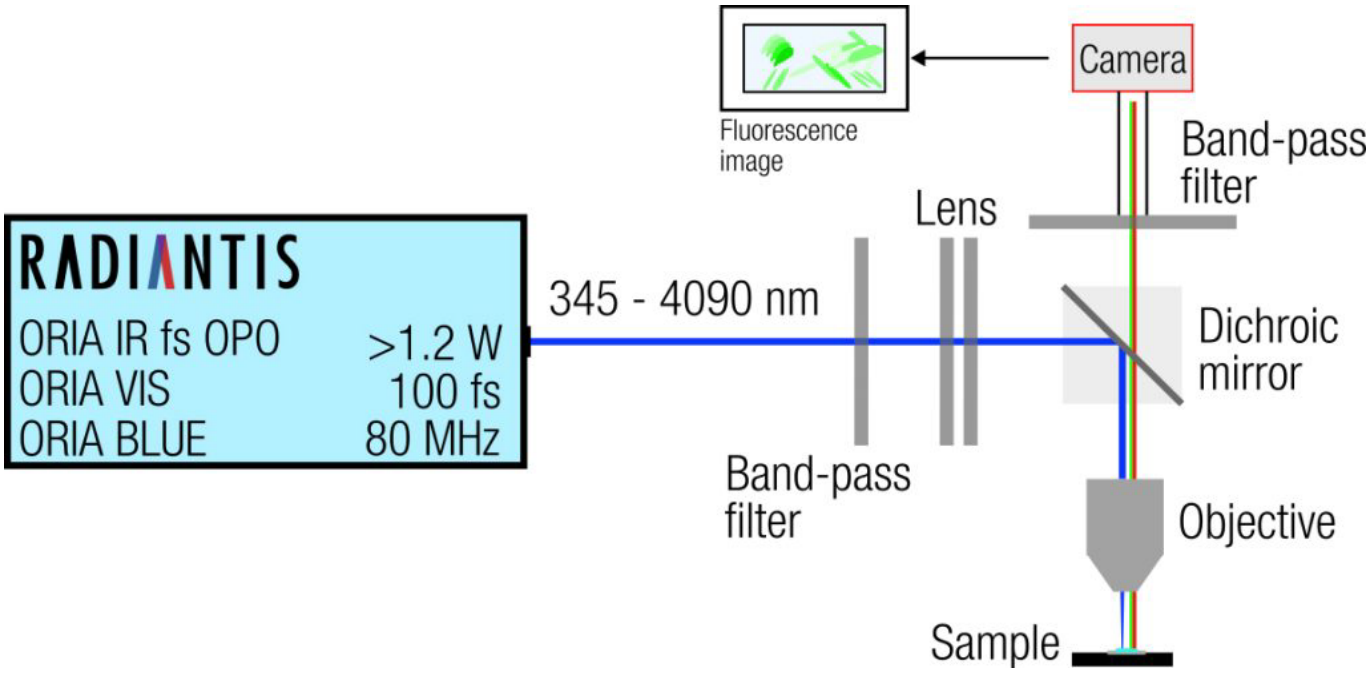

Figure 2: Experimental setup for linear fluorescence microscopy using the Ti:Sapphire-pumped ORIA IR, Oria VIS and Oria BLUE femtosecond OPO laser system.

The advantages of using a broadly tunable laser for fluorescence imaging were tested by analyzing a prepared microscopy slide (FluoCells® prepared slide #2 – Invitrogen). This prepared slide includes three commercially available fluorescent labels: DAPI, Alexa-Fluor 488, and Texas Red.

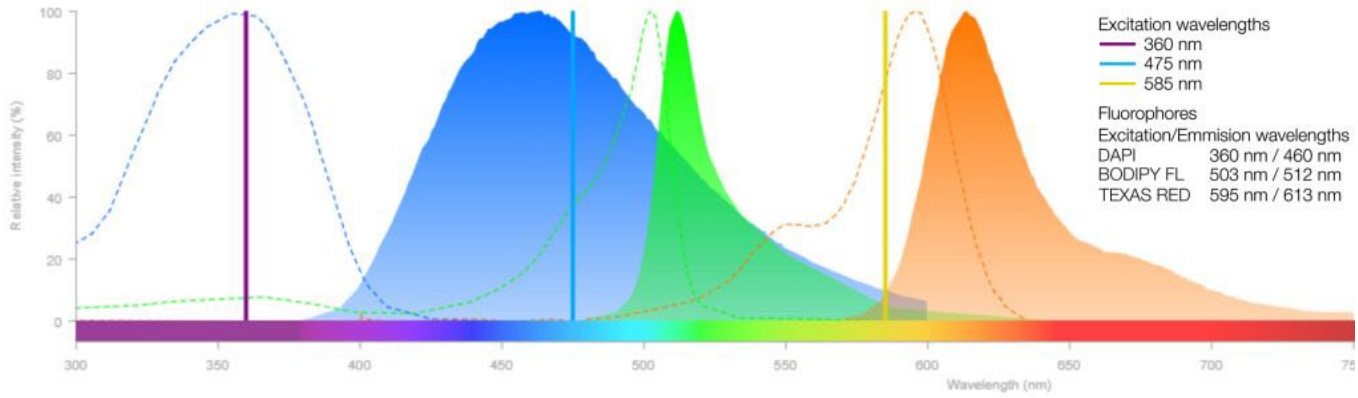

Figure 3: Excitation (dash line) and emission spectra (filled line) of the fluorophores DAPI, BODIPY FL and Texas Red. The vertical-colored lines indicate the excitation wavelengths at which the laser was tuned.

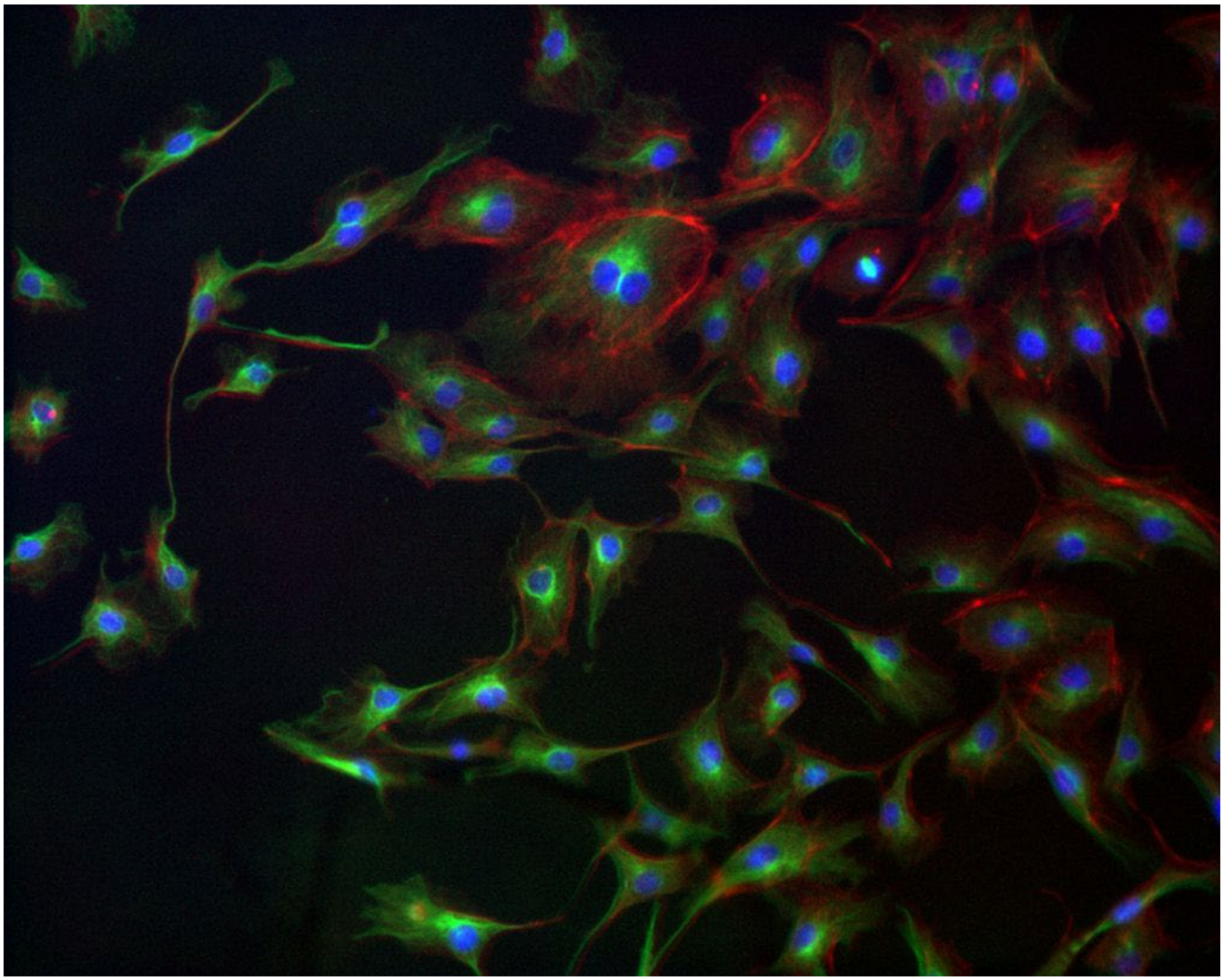

Figure 4: Fluorescence image of bovine pulmonary artery endothelial cells (FluoCells® prepared slide #2 – Invitrogen) using a simple microscope setup in combination with the ORIA IR|VIS|BLUE fs OPO laser from ***Radiantis****.*

In Figure 3, the dotted lines and the filled lines represent the excitation and emission spectral bands of each fluorophore, revealing some overlap. The vertical solid purple, blue, and yellow lines indicate the excitation wavelengths used for selectively targeting these fluorophores. Figure 4 shows the post-processed merged images obtained for the three different fluorophores, revealing in detail not only the position of the fluorophores but also the molecular structure of the cell. DAPI is attached to the nucleus (blue), Texas Red to F-actin (red), and BODIPY FL to microtubules (green).

Typically, a single laser in the visible spectrum is limited to emitting only one wavelength. In fluorescence microscopy, the importance of having multiple wavelengths arises from two primary considerations. Firstly, to effectively excite various molecules that absorb light at distinct wavelengths, it becomes essential to utilize multiple lasers that align with the respective absorption bands of these molecules, typically in the visible spectral range. Secondly, the absorption bands of two different molecules may partially overlap, needing the ability to selectively excite only one of them. This selectivity is achieved by choosing a wavelength that corresponds to the absorption band of the targeted molecule while avoiding exciting the other, in this case 475 nm is selected for exciting the BODIPY FL. Using an Optical Parametric Oscillator (OPO) in linear fluorescence microscopy offers several key advantages. The primary benefit is its broad tunability, allowing researchers to match the excitation wavelength precisely to the fluorophore, improving fluorescence signal strength and reducing background noise. This results in brighter and clearer images without requiring higher laser intensity, which also minimizes photobleaching and phototoxicity.

Their compatibility with advanced imaging techniques like multi-photon microscopy and super-resolution methods into the near IR range expands their applications in complex biological studies, offering high-resolution, high-contrast imaging with reduced sample damage. Moreover, using an ultrashort pulse laser, time-resolved fluorescence measurements can be done, such as FLIM, for instance to deconvolute the fluorescence emission signal in the time domain. This not only enables the observation of ultrafast biological phenomena within cells but also distinguishes between different fluorophores with overlapping excitation bands based on their excited-state decay lifetimes.

In conclusion, broadly tunable Optical Parametric Oscillators (OPOs) are advancing fluorescence microscopy by offering unparalleled wavelength flexibility, minimizing photodamage, and enhancing the quality of multi-color imaging. With their versatility and precision, OPOs, such as the ORIA IR femtosecond OPO laser in combination with the Oria VIS and Oria Blue second harmonic generators, are invaluable tools for advancing biological and cellular research.