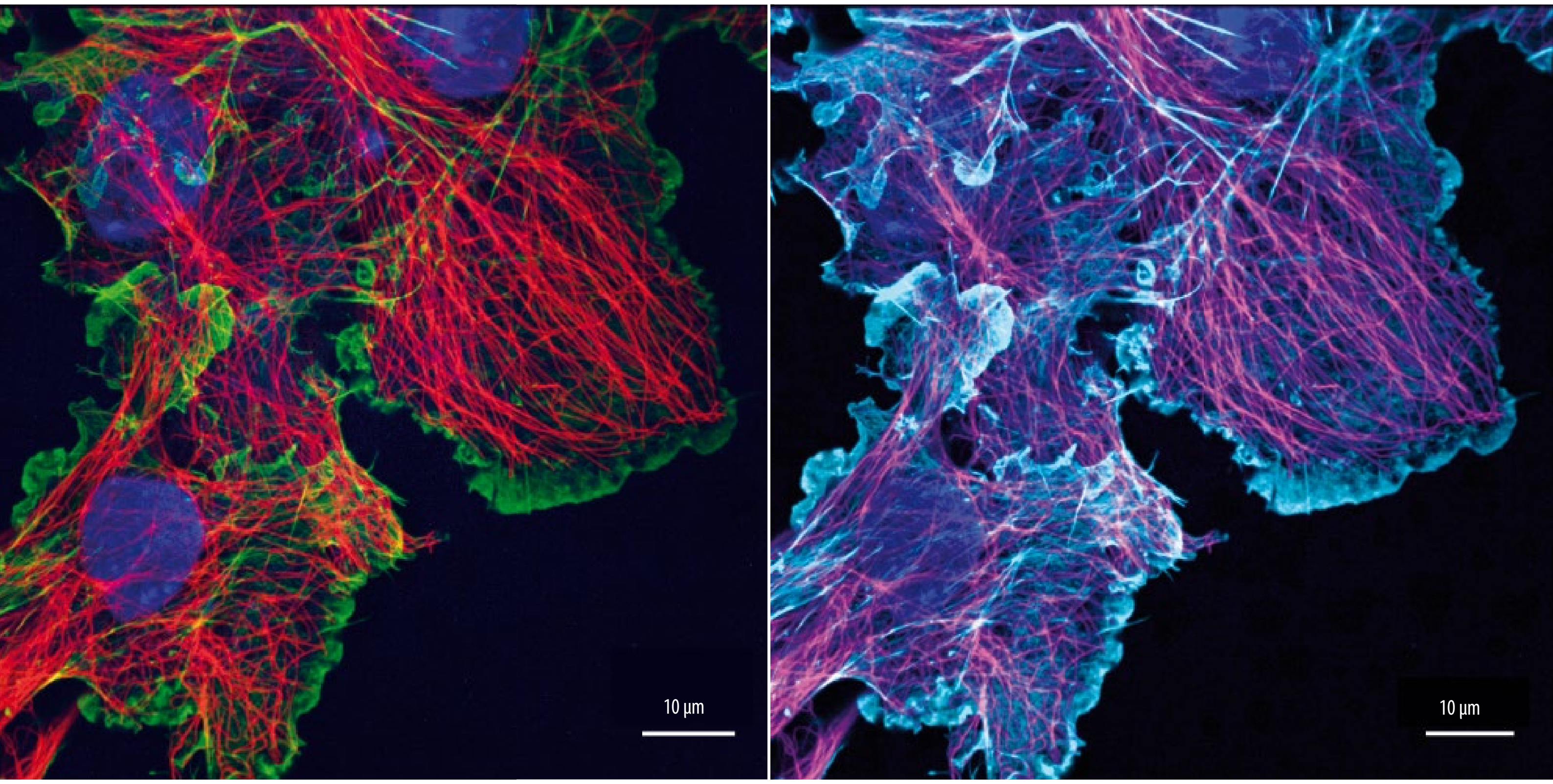


Multiline Lasers for Fluorescence Microscopy

Simplifying multicolor biomedical imaging in compact instrumentation

Melissa Haahr

Fluorescence microscopy instrumentation relies on illumination sources to excite fluorophores. Common illumination sources are LEDs, super-continuum white-light sources, or single-wavelength lasers. Lasers are primarily used for high-resolution and high-throughput imaging techniques, and each wavelength excites a different set of fluorophores. In order to efficiently excite multiple fluorophores, it is necessary to use many single-wavelength lasers in one instrument or experiment. This strengthens the content and quality of results. Along with the advantage of activating more fluorophores comes the challenge of integrating each of the individual wavelengths required.

Typically, there is a need to use between two and eight different lasers. Often this is solved with a laser combiner which includes separate lasers and beam-combining optics. Unfortunately, this can be a large and bulky solution and difficult to keep aligned. In addition, fiber coupling often adds further complexity to the system.

A simplified solution for integrating multiple laser wavelengths into a fluorescence microscope is to use a multiline laser solution. It is now possible to deliver up to four laser colors from one compact and permanently aligned laser package, with one beam output or stacked beams, and an option for direct fiber coupling. The introduction of multiline lasers to fluorescence instrumentation provides a reliable, easy-to-use, and service-free solution to the challenges of including all of the desired wavelengths with reliable, stable performance.

Technological evolution

Over the last decade, fluorescence-based methodology has already been transitioning from bulky gas laser sources into solid-state lasers with a smaller footprint, longer lifetime, and lower maintenance requirements. The development of compact, reliable solid-state lasers was an initial enabling technology for of commercialization microscopy instrumentation and expansion to new markets and applications, accompanied

◂ **Fig. 1** An example of images taken in a single-molecule localization microscopy (SMLM) setup from department of biotechnology & biophysics at Julius-Maximilian-University of Würzburg.The 3-color image shows an african green monkey kidney cell (COS7) with nucleus (blue), microtubules (red/magenta) and the actin sceleton (green/cyan) staining. Recording time 4 s per channel at 2048 × 2048 px field of view.

by parallel improvements in data storage and advanced camera systems, to name a few. While some applications are able to utilize the advancements in LED and super-continuum white-light sources, the high-resolution, high-speed techniques still rely on the high brightness and wavelength precision of lasers.

Currently, many researchers and manufacturers align and integrate individual laser sources for each wavelength on the optical bench or in instrument. These assemblies require additional optics for each laser, also physically separate from the lasers themselves, and all of them need to be aligned with high precision and typically into a fiber delivery system. This design often requires the time and cost of installation and service by a technician from the instrument manufacturer or, perhaps even more costly, the time of a graduate student spent for aligning optics instead of collecting new data. Laser combiners and laser light engines have simplified some of these assemblies substantially. However, they do not eliminate the need for alignment (and re-alignment) over time. Laser combiners can also contribute to the bulkiness of a manufactured solution and can be sensitive to thermomechanical stress causing misalignment.

In addition to the technical requirements of lasers in fluorescence microscopy, there is also a parallel trend towards accessibility of technology and system simplification. As fluorescence imaging or analysis techniques are becoming more common at earlier stages in education and used in a broader range of laboratories, the technology and systems must be useable by operators or students without requiring a high level of expertise in optics. As new techniques are developed for clinical applications, ease-of-use and the ability to commercialize the instrumentation become increasingly important.

The progression of this trend requires a response from the optics industry: While maintaining the highest quality and performance, laser manufacturers must deliver reliable, simple, and cost-effective solutions for both commercial systems and laboratory custom-built instrumentation or basic research.

The use of multiline lasers as an alternative to conventional laser combiners or laser engines solves many of these common pain-points in fluorescence microscopy applications. A “multiline laser” consist of several individual laser wavelengths built into one laser platform and with permanent and stable fixation of all beam alignment optics included on the same platform. The Cobolt Skyra is a totally customizable, permanently aligned multiline laser solution offering up to four individual wavelengths, ranging from 405 nm to 660 nm, in a single laser output.

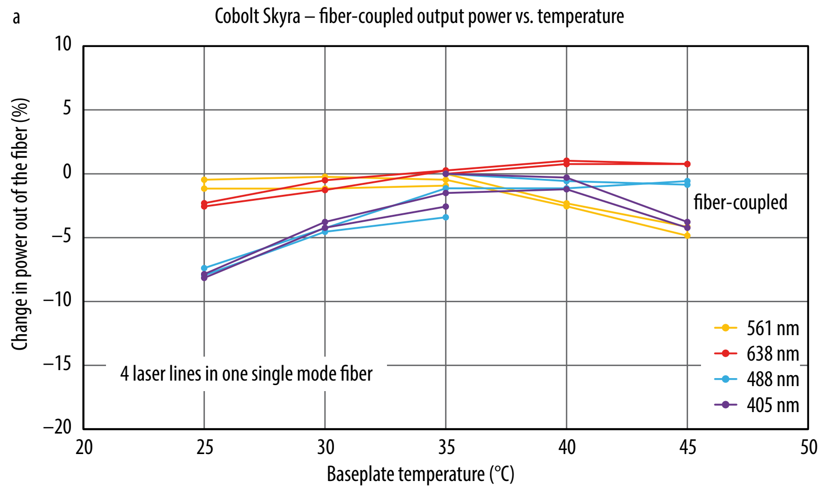
The availability of a compact, easy-to-use, and reliable high-performance multiline laser will assist with the commercialization of new fluorescence-based instrumentation. Moreover, it will further expand existing technologies into laboratories with a lower barrier of entry for both the manufacturer and end-user.

Multiline laser technology

The Cobolt Skyra multiline laser is unique in its design and manufacturing. It is built using patent-pending alignment techniques and utilizing Cobolt’s proprietary HTCure technology. The HTCure technology is based on careful thermo-mechanical matching and high-temperature fixation of miniaturized optics. The lasers are built on a single, temperature-controlled platform for stable operation and protection from thermomechanical misalignment. All the optical elements, including components for beam combining, beam-shaping and alignment are precision-mounted, and the entire package is exposed to high-temperature baking and hermetically sealed. The temperature-stabilized and compact package (meaning short beam paths) provides stable beam-pointing and robustness in varying environmental conditions (**Fig. 2a**). The Cobolt Skyra can be coupled with single-mode, polarization-maintaining fiber coupling directly on the laser head. The output power stability in **Fig. 2a** is measured through the SM/PM fiber, from 20 to 50 °C.

Cobolt’s HTCure technology was an integral part of developing a compact and reliable multiline laser source for

a



b

Fig. 2 Typical power stability out of the SM/PM fiber at each wavelength across the temperature range 20 – 50 °C (a). Cobolt Skyra multiline laser with integrated electronics (dimensions: 70 × 144 × 38 mm, b; source: Hübner Photonics)

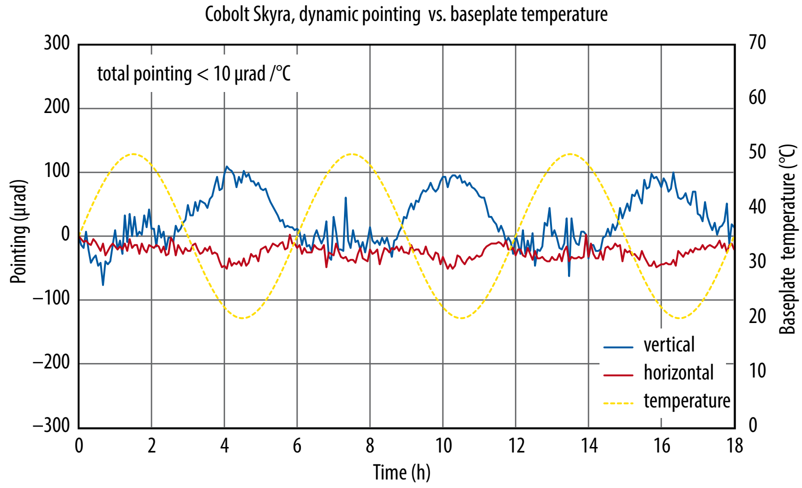


Fig. 3 Vertical and horizontal beam pointing (µrad) over 18 hours continuous laser operation and temperature cycling between 20 °C and 50 °C.

fluorescence microscopy techniques. It eliminated the need to align lasers in the field, by maintaining alignment under various ambient operating conditions, and keeping the laser lines focused into a fiber delivery system. In addition, the control electronics of the multiline laser are integrated directly into the laser head, for a simple, clean, and easily-integrated solution (Fig. 2b).

Different techniques, applications, and day-to-day experiments within fluorescence microscopy for several different projects within a laboratory can have different laser requirements, most of which can be met with a standard or customized variation of a multiline laser source. As standard on Cobolt Skyra, the modulation and control of each wavelength is independent from the others. The controls are compatible with digital and/or analog inputs, as well as software commands via USB. Fast and deep digital modulation up to 5 MHz modulation frequency is possible and 500 kHz in analog modulation.

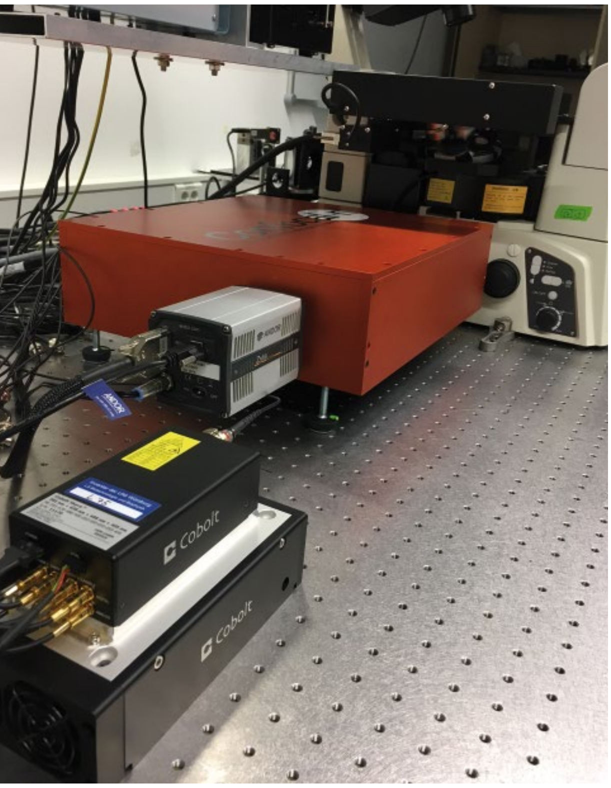


Fig. 4 Cobolt Skyra laser is shown in use in the department of biotechnology & biophysics at Julius-Maximilian-University of Würzburg [1].

Programming the laser wavelengths for on/off operation or with different modulation sequences, with only one USB connection, greatly simplifies the equipment needed to integrate and operate the laser. For duty cycles up to 1 ms modulation signals can be sent directly through software and eliminate the need for an external function generator. Individual laser communication can simply be replaced by one USB connection, and still allow for individual laser control. Commands can be sent to the Skyra laser either with Cobolt's own software, Cobolt Monitor, or through a custom-built program utilizing software such as National Instruments LabView. Furthermore, the software compatibility allows for remote control as well as remote servicing of the laser, further reducing the cost of ownership.

In addition to the inherent flexibility of multiline lasers in the laboratory or commercial instruments, custom wavelength combinations are also available, both with or without direct fiber coupling. By including both direct-diode and diode-pumped solid state laser technology on the multiline laser platform, a wide range of wavelengths become available. The Skyra can include up to four wavelengths, within the range of 405 nm to 660 nm with beam position overlap <50 µm at the exit and pointing stability <10 µrad/°C over a temperature range of 20 °C to 50 °C (Fig. 3). The output beams of the Cobolt Skyra can be collinear and coupled into single mode fibers for convenient launching into microscope set ups or tailored to form stacked light sheets at a precisely defined location in front of the laser for direct alignment to, for example, a flow cell in a cytometer.

In the lab

Some of the earliest users of the Skyra in academia have utilized the technology to equip laboratories with a powerful tool for multiple types of microscopy techniques. One such laboratory is that of Markus Sauer at the department of biotechnology and biophysics at Julius-Maximilian-University of Würzburg. Researchers in Prof. Sauer's lab are focusing on single molecule sensitive fluorescence spectroscopy and imaging techniques, including super-resolution microscopy and its applications in biomedical sciences. The Skyra has, for example, been used in a single-molecule localization microscopy (SMLM) setup to gain new insights into the organization of proteins within a cell (Fig. 4). The system provides images with spatial resolution nearing the molecular level from which quantitative biological data can be extracted (Fig. 1). The Skyra was an economical, high-performing, and easy-to-use solution in their instrumentation, helping to move research along at a faster pace with consistent results [1].

Additional techniques for the use of multiline lasers are in the field of cancer diagnostics and the progression towards increasing fluorescence instrumentation in clinical settings. The barrier of developing such suitable instrumentation and achieving clinical certification is high, but a critical step is creating advanced instrumentation that also has the capability to be commercialized and accessible. The first challenge is to develop the technique, but it is often followed by a second challenge of making that new technology dependable and user friendly.

A team from Dr. Jonathan Liu's laboratory at the University of Washington has recently developed a cutting-edge open-top light sheet microscope for fast, non-destructive, slide-free, 3D pathology [2]. The technique rapidly images 3D biological samples, without slicing the tissue-sample as in traditional pathology techniques. A unique application for this technology can be found in prostate needle-core biopsies and cancer diagnosis. Furthermore, Dr. Liu and his team have continued to drive their technology towards commercialization. The use of an easy to control, compact, and permanently aligned multiline laser assisted in the simplification of the optical assembly in their innovative instrument design [3].

Outlook

Fluorescence imaging is a key technique in both biomedical research and clinical diagnosis. Fluorescence microscopes for high-resolution and high-throughput multifluorophore imaging typically rely on the use of several individual laser sources at different wavelengths within the same instrument. Traditionally, these lasers have been coupled into the microscopes through laser combiners which have added bulk, cost, and alignment complexity.

Multiline laser solutions are an attractive alternative to laser combiners to simplify fluorescence imaging instrumentation and, furthermore, aid in the process of commercialization for new, cutting-edge imaging systems for clinical use. Multiline lasers enable smaller and more cost-efficient instruments which are much easier to manufacture and maintain. This supports the strive for bringing more advanced laser-based instrumentation into research and clinical settings for improved medical diagnostics and further development of new analytical techniques.

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[2] *A. K. Glaser* et al.: Light-sheet microscopy for slide-free non-destructive pathology of large clinical specimens. Nat. Biomed. Eng. **1** (2017) 0084.

[3] *A. Glaser* et al.: https://lightspeedmicro.com/ January 2019.

Company

Cobolt

Cobolt is a part of HÜBNER Photonics and is based in Stockholm, Sweden. HÜBNER Photonics offers a wide range of Cobolt lasers, the tunable cw-laser: C-WAVE, plus new solutions in the fields of terahertz imaging and spectroscopy, high frequency components, and radar systems. Cobolt supplies high performance CW and Q-switched lasers for advanced bioimaging, spectroscopy, and interferometry applications. The Cobolt lasers are manufactured in compact and hermetically sealed packages using proprietary HTCure technology for robustness and reliability.

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