Fluorescence microscopy instrumentation simplified using novel multi-line lasers

Melissa Haahr, Elizabeth Illy, Håkan Karlsson, Magnus Rådmark
Cobolt AB, Vretenvägen 13, Solna, Sweden, 17145

ABSTRACT

Conventional fluorescence-based bio-instrumentation equipment typically uses multiple individual lasers combined through optical elements into one beam or an optical fiber. The systems can become bulky, costly to manufacture, and challenging to keep aligned. An extremely compact, permanently aligned, and service-free multi-line laser device can reduce the size and cost of these systems for fluorescence-based research. Removing the complexity of integrating individual lasers with a multi-line solution makes the techniques more cost-efficient, user-friendly, and accessible for all levels of researchers.

Here we demonstrate how multi-line lasers are integrated into fluorescence-based instrumentation to simplify experiments without compromising the quality of the results. Integrated electronics, software interfacing, and individual control of each laser-line allow for full flexibility to tailor the laser for the exact experimental needs. Applications include fluorescence microscopy (SIM, TIRF, STED), confocal microscopy, flow cytometry, and combined techniques in research laboratory environments.

The Cobolt Skyra™ multi-line laser is an extremely compact laser device (14.4 cm x 7.0 cm x 3.8 cm) with up to 4 laser lines in one permanently aligned output beam. All optical elements are assembled onto one ultra-stable platform, using patented HTCure™ technology developed by Cobolt, with high precision and permanent alignment. In addition, the multi-line laser can be customized with any combination of more than 14 colors, ranging from 405nm to 660nm, as well as fiber coupling.

Keywords: Fluorescence microscopy, multi-line laser, lasers for microscopy, laser combiners, light-sheet microscopy, clinical diagnostics, CW lasers, light engines, cell imaging

1. INTRODUCTION

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, laser combiners can be a large and bulky solution, and difficult to keep aligned. In addition, fiber coupling often adds further complexity to the optical system.

A simplified solution for integrating multiple laser wavelengths into a fluorescence microscope is to use a multi-line 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 multi-line 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.

In addition to the simplification of laser integration, a multi-line laser also brings the advantage of customization and unique control capabilities. Across different techniques, laboratories, or even individual experiments, there are various

requirements on the colors, power output, modulation, and beam configuration; all of which can now be accommodated with one ultra-stable solution.

A compact, multi-line laser that is customizable but easy to use, while maintaining the essential laser performance and specifications required in fluorescence microscopy has suitability in a broad range of techniques and applications. Here we will present how the Cobolt Skyra™ multi-line laser is beneficial in laboratory research, and in moving newly-developed techniques from the lab bench into commercially-viable products. In addition, we will comment on how a reliable, permanently-aligned, and easy-to-use multi-line laser can simplify the instrumentation necessary for clinical diagnostics.

2. MOTIVATION

The development of the Cobolt Skyra™ was motivated by the scientific and practical requirements of fluorescence microscopy applications for both research and instrument manufacturing.

Over the last decade, the industry 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 commercialization and expansion to new markets and applications, accompanied 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 the instrument. These assemblies require additional optics for each laser, also physically separate from the lasers themselves, and all of which 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 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. Highly advanced cellular information is now available at the early levels of research or education and is expected to come quickly and with ease. 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 multi-line lasers as an alternative to conventional laser combiners or laser engines solves many of these common pain-points in fluorescence microscopy applications. A “multi-line laser” is several individual laser wavelengths built into one laser package and with permanent and stable fixation of all beam alignment optics included the same package. The Cobolt Skyra™ is a totally customizable, permanently aligned multi-line laser solution offering up to four individual wavelengths, ranging from 405nm to 660nm, in a single laser output.

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

3. ENABLING TECHNOLOGY

The Cobolt Skyra™ multi-line 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 miniaturised optics. The lasers are built on a single, temperature-controlled platform for stable operation and protection from thermomechanical mis-alignment. 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) provide stable beam-pointing and robustness in varying environmental conditions (Figure 1a). The Cobolt Skyra™ can be coupled with single-mode, polarization-maintaining fiber coupling directly on the laser head. The output power stability in figure 1a below is measured through the SM/PM fiber, from 20 to 50^0C.

Cobolt's HTCure™ technology was an integral part of developing a compact and reliable multi-line laser source for 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 multi-line laser are integrated directly into the laser head, for a simple, clean, and easily-integrated solution (Figure 1b).

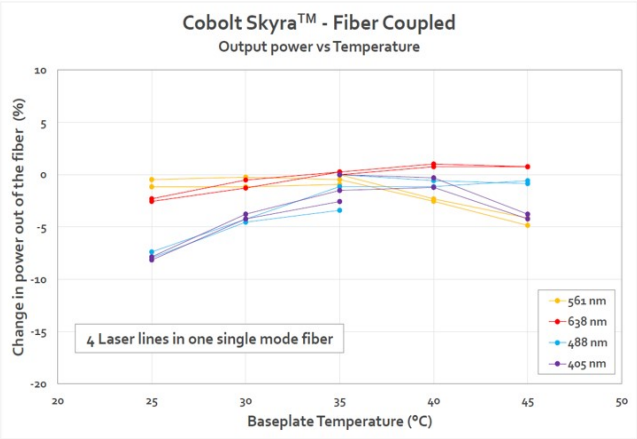


Figure 1a: Left – typical power stability out of the SM/PM fiber at each wavelength across the temperature range 20-50ºC.
Figure 1b: Right – Cobolt Skyra™ multi-line laser with integrated electronics (Dimensions: 70 x 144 x 38mm).

4. A CUSTOMIZABLE SOLUTION

Different techniques, applications, and day-to-day experiments within fluorescence microscopy or a single laboratory can have differnt laser requirements, most of which can be met with a standard or customized variation of a multi-line 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 5MHz modulation frequency is possible (figure 2) and 500kHz in analog modulation.

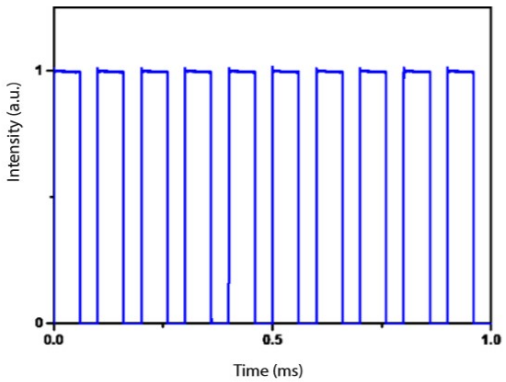
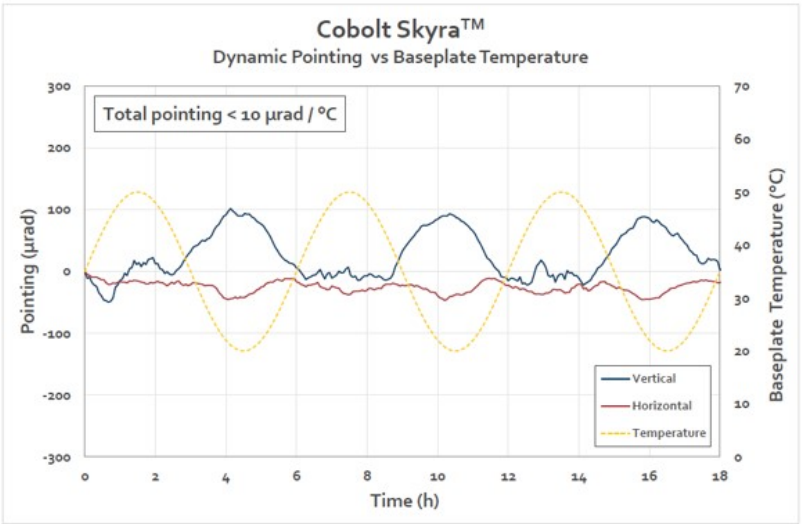


Figure 2: Typical pulse train with sub-microsecond pulses of Cobolt 473nm diode laser with direct digital modulation.

Programming the laser wavelengths for on/off operation or with different modulation sequences, often with only one USB connection, greatly simplifies the equipment needed to integrate and operate the laser. For duty cycles up to 1ms, modulation signals can be sent directly through software via one USB connection and eliminates the need for an external function generator. Individual laser communication can be simply replaced with one USB connection, and still allowing for individual laser control. Commands can be sent to the Cobolt Skyra™ 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 multi-line lasers in the laboratory or commercial instrument, custom wavelength combinations are also available, with or without direct fiber coupling. By including both direct-diode and diode pumped solid state laser technology on the multi-line laser platform, a wide range of wavelengths are available. The Cobolt Skyra™ can include up to four wavelengths, within the range of 405nm to 660nm with beam position overlap <50um at the exit and pointing stability <10urad/°C over a temperature range of 20°C to 50°C (Figure 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.



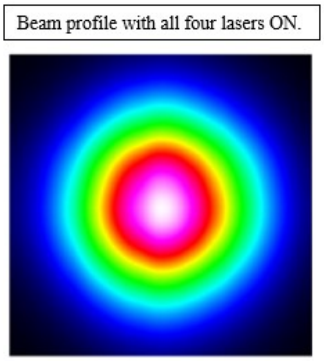


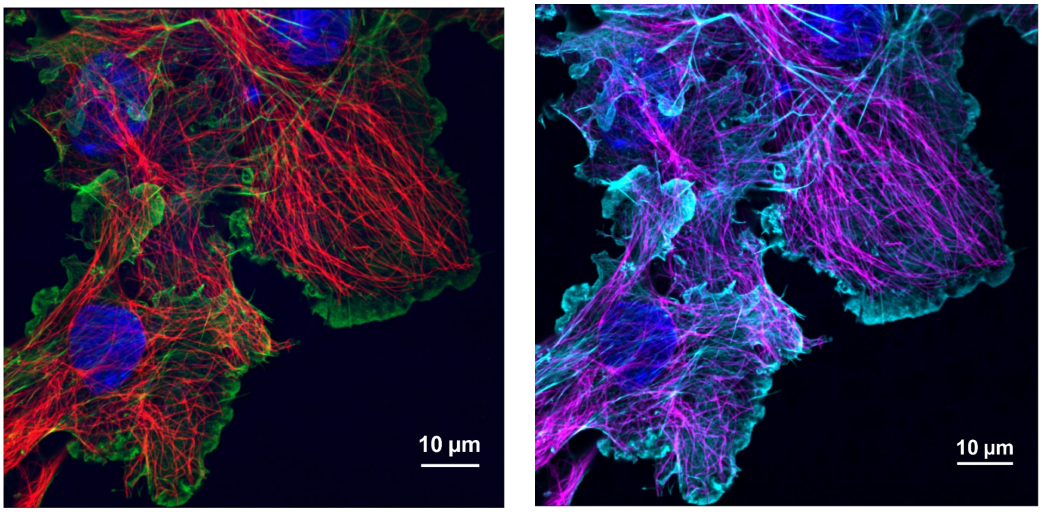
Figure 3a (left): Vertical and horizontal beam pointing (urad) over 18 hours continuous laser operation and temperature cycling between 20°C to 50°C.

Figure 3b (right): Beam profile of typical 4-line Cobolt Skyra™ demonstrating Gaussian beam overlap.

5. APPLICATIONS

Some of the earliest users of the Cobolt 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 Prof. Dr. Markus Sauer at the Department of Biotechnology and Biophysics at Julius-Maximilian-University of Würzburg. Researchers in Prof. Dr. Markus 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 Cobolt Skyra™ laser 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. The system provides images with spatial resolution nearing the molecular level, from which quantitative biological data can be extracted (Figure 4a, 4b). The Cobolt 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]

(4a)



(4b)

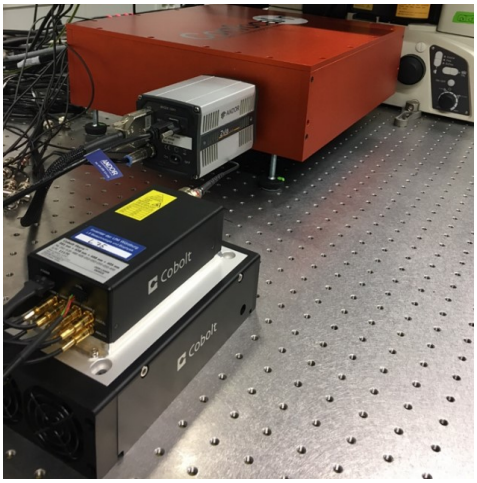


Figure 4a. An example of an images taken in a single-molecule localization microscopy (SMLM) setup from Department of Biotechnology & Biophysics at Julius-Maximilian-University of Würzburg.The 3-color image shows african green monkey kidney cell (COS7) with nucleus (blue), microtubules (red/magenta) and the actin sceleton (green/cyan) staining. Recording time 4s per channel at 2048x2048px field of view.

Figure 4b. Cobolt Skyra™ laser is shown in use in the Department of Biotechnology & Biophysics at Julius-Maximilian-University of Würzburg.[1]

Another extremely interesting technique for use of multi-line lasers is 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 is applied to prostate needle-core biopsies and cancer diagnosis. Furthermore, Dr. Liu and his team have continued to drive their technology towards commercialization[3]. The use of an easy to control, compact, and permanently aligned multi-line laser assisted in the simplifying of the optical assembly in their innovative instrument design[3].

6. CONCLUSION AND OUTLOOK

Fluorescence imaging is a key technique in both biomedical research and clinical diagnosis. Fluorescence microscopes for high-resolution and high-throughput multi-fluorophore 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.

In this work, we have shown that multi-line 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. One such multi-line laser source is the Cobolt Skyra™, which is a permanently aligned, compact, and easy-to-use laser with up to four different laser lines integrated into one single hermetically sealed package. The Cobolt Skyra™ multi-line laser concept is based on the HTCure™ laser manufacturing technology, in which miniaturized optics are fixed onto a thermo-mechanically stable platform using a high-temperature baking process.

The very precise, stable and compact beam alignment enabled by the HTCure™ technology allows for direct integration of the laser source into the instrument without the need for further alignment of individual lasers or optics for beam shaping or combining. In this way, the Cobolt Skyra concept can transform the way laser-based multi-colour bioinstrumentation is designed and manufactured. It enables 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.

In two examples we the have demonstrated how multi-line lasers can simplify the use of a research laboratory fluorescence microscopy set-up and facilitate commercialization of a new fluorescence microscopy technology targeting clinical diagnosis applications. Looking ahead, commercially established techniques such as flow cytometry could benefit from the use of multi-line lasers for direct integration with the flow cell and permanent alignment of the laser source. Optical manufacturing technology such as HTCure™ could further be utilized for compact and reliable optical assembly in fluorescence instrumentation, as simplification of high-performance lasers and optics becomes an integral part of analytical instrumentation development and improvement.

7. REFERENCES

[1] M. Sauer and M. Heilemann, *Chem. Rev.*, 117, 7478−7509 (2017); doi:10.1021/acs.chemrev.6b00667.
[2] Glaser, A. K. et al. Light-sheet microscopy for slide-free non-destructive pathology of large clinical specimens. Nat. Biomed. Eng. 1, 0084 (2017).
[3] Glaser, A., Reder, N., Liu, J., Buckley, S., True, L. https://lightspeedmicro.com/ January 2019.