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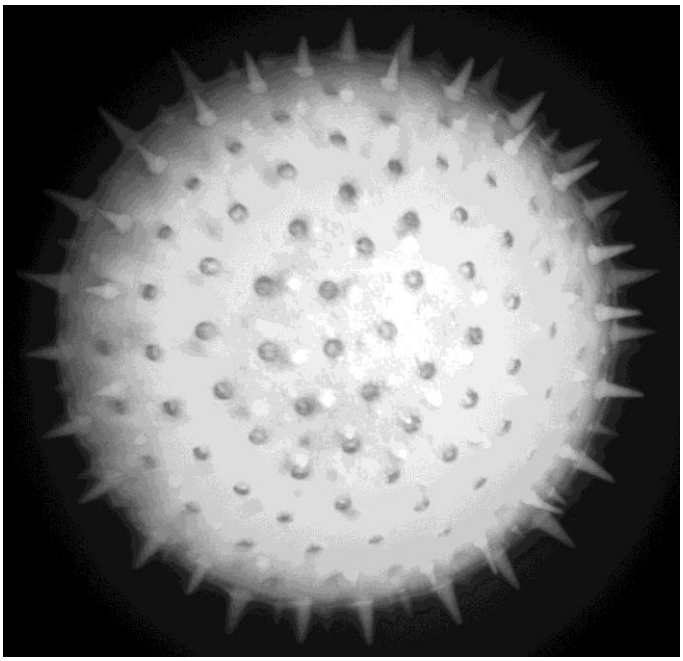
Application Note :

Optical focusing in microscopy with

Optotune's focus tunable lens EL-10-30



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Summary

Featuring a large aperture, fast response and actuation times, and good optical quality the EL-10-30 offers considerable prospects for various applications in microscopy. In this application note, we discuss the implementation of electrically tunable lenses (ETLs) for fast axial focusing in three different microscopy methods: (1) Classical wide-field fluorescence microscopy, (2) fluorescence confocal microscopy and (3) two-photon microscopy. For a complementary introduction detailing these techniques, we would like to refer to http://micro.magnet.fsu.edu/primer/index.html.

General considerations and typical implementations

ETLs can be used for different applications in microscopy. These include specialized tunable illumination systems as well as electrically controlled zoom optics, among many others. In this application note, we discuss the use of ETLs for focusing along the optical axis exclusively. Depending on the implementation of the ETL and the optical performance requirements, axial focusing ranges of 30-700 microns can be achieved. Nevertheless, most of the discussed technical details for using ETLs in microscopy apply for other applications as well. In standard microscopes, axial focusing is usually achieved by moving either the specimen on a z-stage or the microscope objective. A common alternative solution for precise focusing is the use of piezo-actuated objective mounts. However, these focusing techniques are based on mechanical axial movements relative to the specimen. Movement-free and even faster focusing can be realized if optical focusing schemes are used. A convenient solution to realize optical focusing is the inclusion of ETLs in the optical pathway of the microscope. Modern microscope objectives have infinity-corrected optics, which means that light originating from a point within the focal plane of the objective is not focused to a point within the image plane, but emerges from the objective as a parallel beam of light (focused to infinity) (Figure 1). An additional tube lens is required to form a real image on a detector.

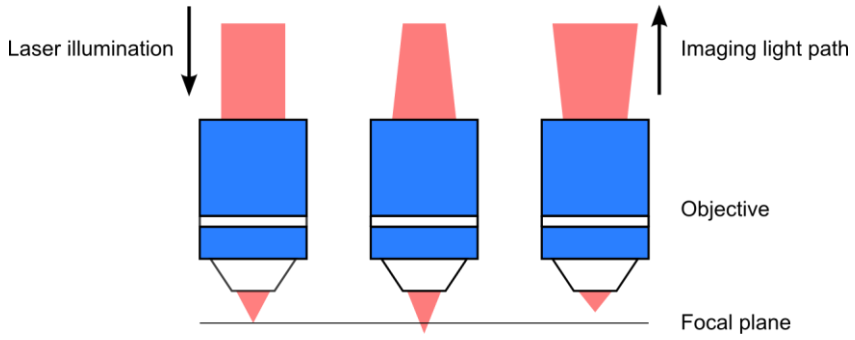

Figure 1: An infinity corrected objective focuses a parallel beam of light to a spot on its focal plane and vice versa during imaging. A point source located below or above the nominal focal plane leads to a converging or diverging beam, respectively.

If the object or light source is moved closer to the objective front lens, the objective yields a diverging beam, whereas an object placed further away than the nominal working distance results in a converging beam. These conditions can be considered in the other way round: If a tunable optical component can be used to create a diverging or converging beam at the objective, the microscope can be focused along the optical axis. This applies both to illumination and imaging light paths through the objective as in a laser scanning microscope.

As the EL-10-30 can only create a tunable converging beam, it has to be paired with an offset lens of negative focal length to achieve a tuning range from negative to positive focal lengths (Figure 2). We combined plano-concave singlets with a focal length of -100 mm with an EL-10-30 (low dispersion variant, focal length range of

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50-200 mm). The combination of ETL and offset lens (OL) can be mounted in a custom lens holder or in a standard 30 mm lens tube.

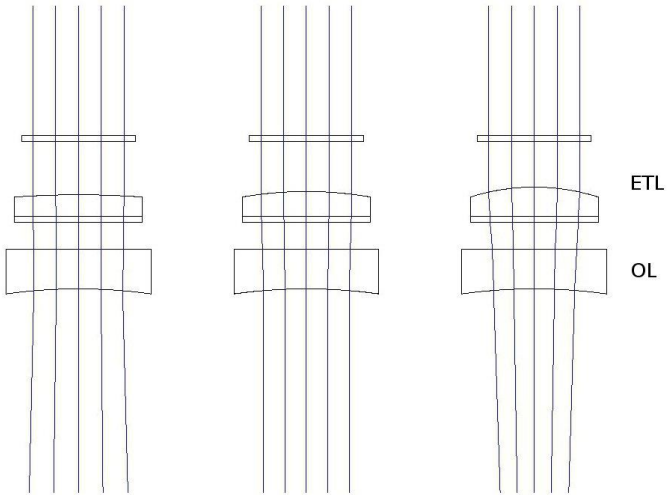

Figure 2: ZEMAX model of the EL-10-30 (ETL) and an offset lens (OL) with negative focal length (f= -100 mm, Thorlabs LC 4232). Please note the change in curvature of the tunable surface and the resulting change in beam divergence. A ZEMAX modeling package of the EL-10-30 is available for download at www.optotune.com.

The easiest way to achieve optical focusing is to mount the ETL/OL assembly as close to the objective as possible (Figure 3). For example, the ETL and OL can be mounted in a lens tube with two RMS threads and inserted between objective revolver and objective - the same way a piezoelectric focusing device is inserted.

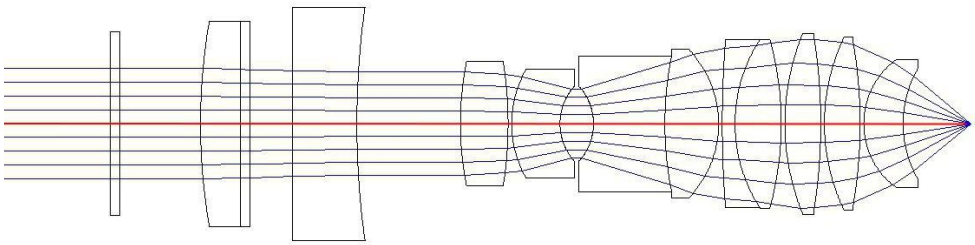

Figure 3: ETL/OL assembly combined with a microscope objective (USP 4231637). The axial length of the entire system is about 50 mm. In this system, a shift of the focal length of the EL-10-30 from 150 to 80 mm leads to an axial focus shift of -50 to +50 micron around the nominal working distance. The focal length of the objective is 4.4 mm.

Positioning the ETL and OL close to the objective rear stop is straightforward and simple to implement, it is however a suboptimal location for optical focusing and not suitable for all imaging applications. Most microscope objectives are telecentric, meaning that the central ray in each bundle (e.g. the on-axis ray in Figure 3, red), also known as chief ray, propagates along to the optical axis after leaving the objective front lens. Focusing with the ETL placed close to the objective leads to non-telecentric conditions (Figure 4). The result is a change in field-of-view (FOV) size or magnification if the axial focus position changes. If the desired focus shifts are small (< 50 micron), this can be tolerated. Alternatively, the ETL/OL assembly can to be positioned at a the conjugate pupil plane of the microscope objective by means of a 4f relay system (e.g. a 4f-system composed of two achromats) as most objectives have an inaccessible stop (Figure 5).

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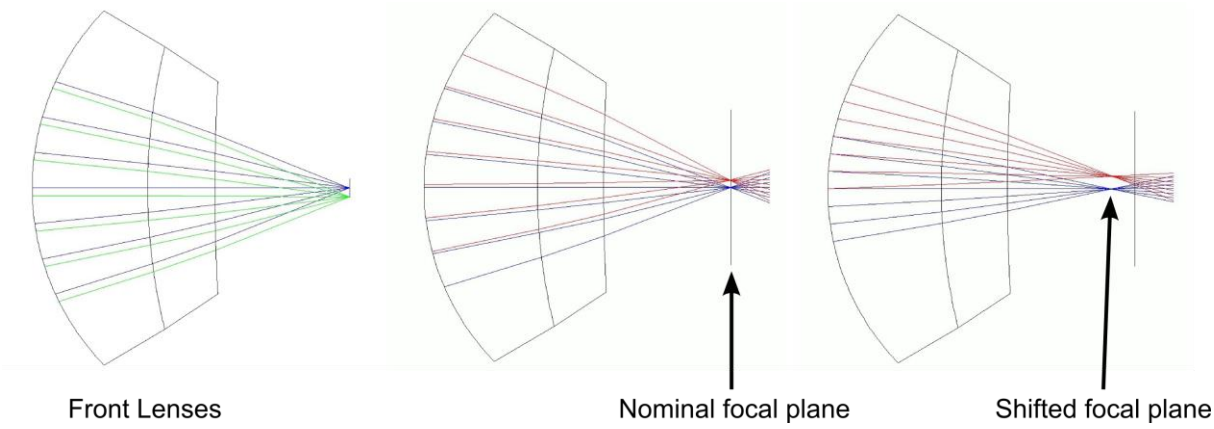

Figure 4: Microscope objective front lenses with two ray bundles focusing at different lateral points at the sample. In the left panel, two bundles are shown under telecentric conditions – the central green (chief) ray propagates parallel to the optical axis (blue chief ray). In the center and right panels, focusing with the same objective and the ETL/OL inserted close to its rear stop is shown. The chief ray of the oblique (red) ray bundle does not propagate parallel to the optical axis, as a result, the distance between the red and blue foci increases if the focal plane is shifted closer to the objective (leading to a change in magnification or FOV size).

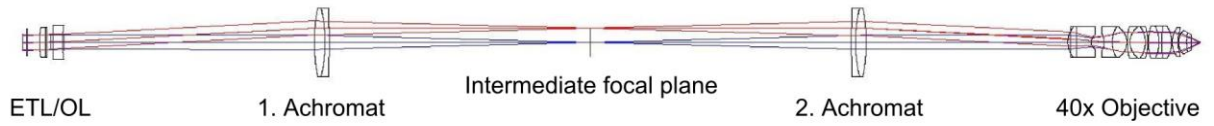

Figure 5: Example layout of a 4f-relay system to preserve telecentric imaging conditions. The ETL/OL assembly (on the left side) is placed at a conjugate pupil plane of the objective (Japanese Patent 8–292374; right side) by a 4f-system composed of two f=100 mm achromats in the middle section. The pupil (intersection of red and blue light bundles) is reimaged into the objective. The total length of the system is about 380 mm. To create an image, a tube lens is necessary on the left side of the system. During ETL-based focusing, the intermediate focal plane is shifted.

Most commercial microscopes, however, do not allow a relay system to be inserted into the light path. In this case, placing the relay system at a camera or laser port might be an option.

Controlling the EL-10-30

The EL-10-30 can be easily computer-controlled by using a precision constant current driver for laser diodes (e.g. Edmund Optics NT56-804, Thorlabs LD1255R) and a 0-250 mA programmable analog output. For simple focusing applications, a calibrated lookup-table relating control current to focus positions is sufficient (Please see Figure 15).

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The two types of implementation are summarized below:

Type of implementation	ETL/OL close to the rear stop	ETL/OL at a conjugate pupil
Advantages	Straightforward to implement, especially in commercial microscopes	No change in the FOV size / magnification over the focusing range
Disadvantages	Change of FOV size / magnification over the focusing range. Change in numerical aperture and thus resolution over the focusing range	Depending on the operating conditions, a change in numerical aperture can occur due to vignetting at the front lens. Needs a relay system, therefore less straightforward to implement, needs space
Typical application	Confocal microscopy (approx . 50 micron range) Two-photon microscopy (max. 700 micron range)	Ideal for custom-built microscopes. Beneficial for visual use.

Application example: Wide-field microscopy

For visual use, a change in the FOV or magnification while focusing is confusing (as its visual appearance corresponds to a zoom effect). For small focusing increments (a few microns), this can be tolerated, otherwise, the effect becomes disturbing. In this case, placing the ETL/OL assembly at a conjugate pupil position is necessary. In most microscopes, a custom-built module which contains additional relay optics and the ETL/OL assembly has to be inserted into the optical path. In typical upright microscopes, the stop position is located inside the objective and not accessible, thus necessitating a relay system. In inverted microscopes, however, a conjugated pupil is often formed by the optics inside the microscope body and in certain types of these microscopes, this pupil is accessible and located in a vertical portion of the optical path, making it ideal for insertion of an ETL-based focusing system. One such microscope is the 1980s Zeiss Axiovert 35 as sketched in Figure 6.

Setup

The ETL/OL assembly is placed close to the pupil by inserting both components into a 30 mm lens tube and attaching it to an optical rail (Figure 8). In order to localize the pupil position, a simple trick can be used: Phase masks that are used for phase microscopy are reimaged into the conjugate pupil plane. The position where a sharp image of a phase mask in the condenser or objective is formed is the ideal position for the ETL (Inset in Figure 8). This type of ETL-based focusing has the additional advantage that the ETL can be used with a wide range of objectives. Depending on the objective, the optimum location of the ETL and OL may be shifted, as some types of objectives differ in their pupil imaging properties.

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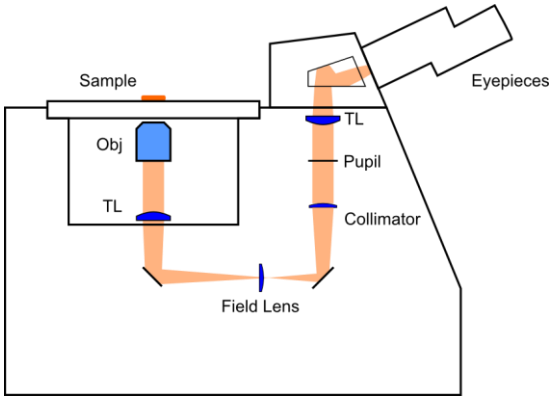

Figure 6: Optical path of the Axiovert 35 microscope. The ETL/OL assembly can be placed at the pupil without inserting an additional relay system. TL: Tube lens.

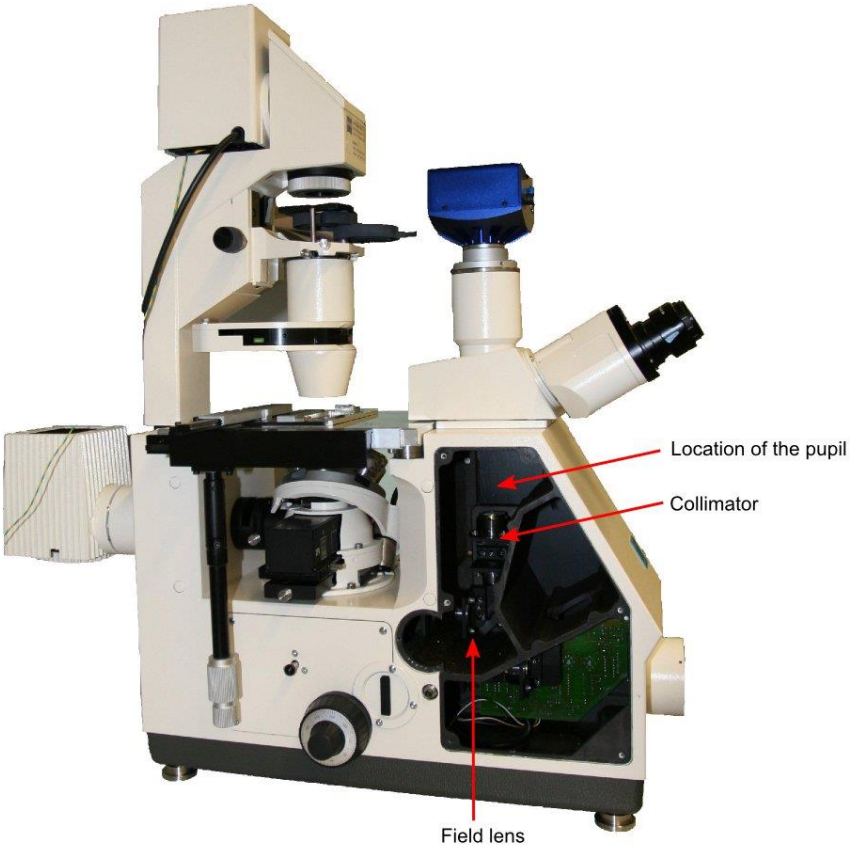

Figure 7: Axiovert 35 microscope with removed side-cover to access the pupil. Some of the optical components shown in Figure 6 are highlighted.

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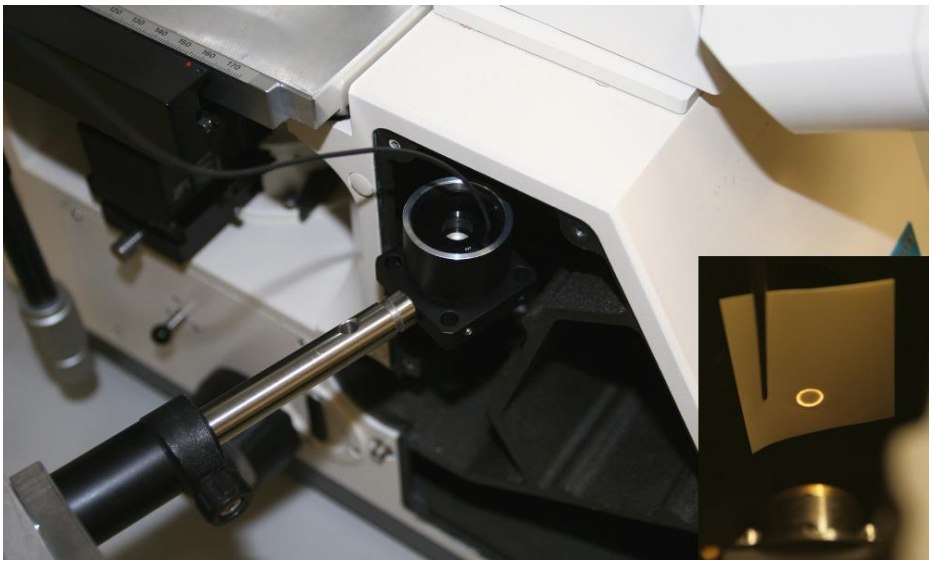

Figure 8: Insertion of the ETL/OL assembly at the conjugated pupil position. ETL and OL are mounted on a post that is attached to an optical rail. Inset: The conjugate pupil position can be found by looking for a sharp image of a phase ring.

Results

In combination with a 40x NA 0.6 Objective (Zeiss LD Achroplan 40x / 0.6 Korr Ph2), defocusing ranges up to 120 micron can be achieved (The correction ring was set to a fixed position). Example images of z-stack acquired with ETL-focusing of a group of pollen grains (epifluorescence mode) are shown in Figure 9.

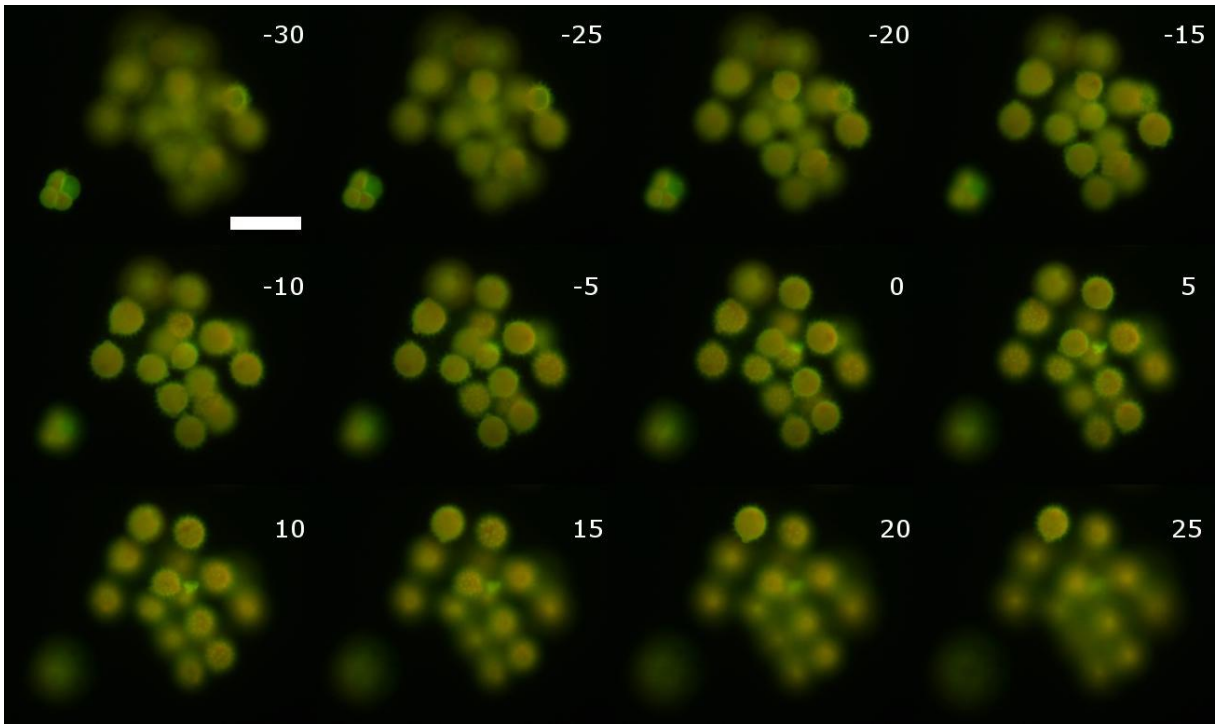

Figure 9: ETL-based focusing through a group of pollen grains. The images range from -30 to + 25 micron relative to the nominal image plane. Due to the position of the ETL at a conjugate pupil there is no change in magnification. Scale bar: 100 micron.

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Application example: Confocal microscopy

One of the most important microscopy techniques is confocal microscopy with a large variety of applications in cell biology, single molecule physics and many other fields. For a more detailed description of this microscopy method and different types of confocal microscopes, please refer to http://micro.magnet.fsu.edu/primer/index.html.

Setup

Standard confocal microscopes are highly integrated systems without the possibility to access or insert optics in most parts of the optical path. Depending on the specific microscope, different solutions of inserting the ETL/OL assembly are possible. One possibility is to mount the ETL/OL assembly in a custom filter cube in the filter turret of the microscope stand if the filter cubes are not necessary for confocal imaging. Alternatively, the ETL can be mounted in a relay system (Figure 10).

The microscope we used[1] (Figure 11) is a spinning disk confocal with the confocal unit (Yokogawa CSU X1) and CCD camera attached to the side port of an Olympus IX-71. This microscope configuration allows the insertion of a modified original filter-cube containing the ETL and OL into the filter turret without the need for extensive modifications of the microscope. During operation, the ETL can be removed from the optical path simply by rotating the filter turret to an empty position.

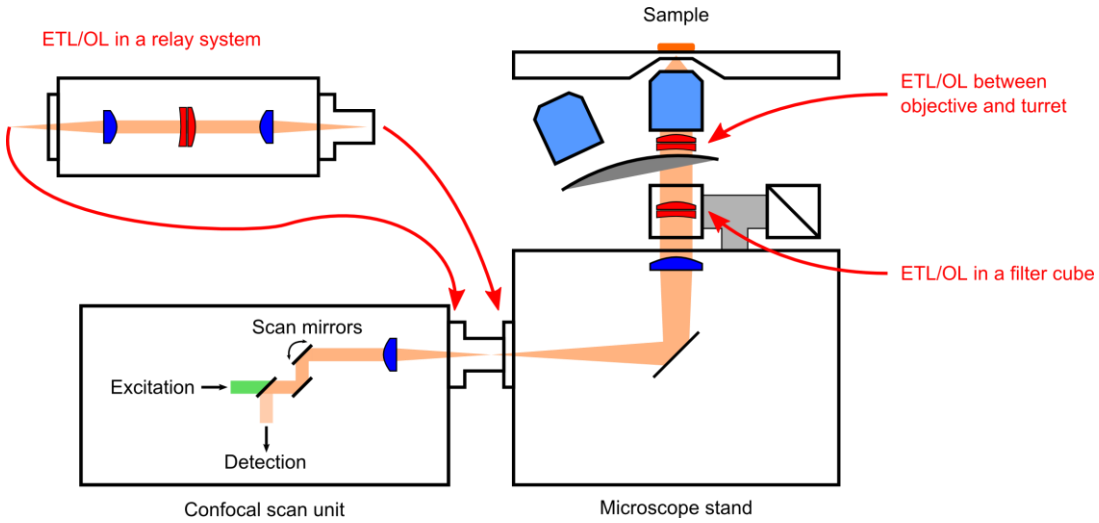

Figure 10: Three different options to implement ETL-based focusing in a typical inverted laser scanning microscope. (1) The ETL/OL may be placed between microscope objective and objective turret like a piezo-actuated focusing device. (2) If possible, ETL and OL can be mounted in a custom filter cube. Both of these options share the disadvantage of a possibly significant change in magnification during focusing. (3) To avoid this, a relay system can be inserted between the confocal scan unit and the microscope stand. For optimum system performance, the ETL should be in a vertical portion of the optical path – otherwise, asymmetrical gravity-induced deformations of the tunable membrane might lead to image degradation.

[1] We thank the lab of Helge Ewers at ETH Zurich (http://www.neuro.nano-optics.ethz.ch/) for the opportunity to use the EL-10-30 in combination with the spinning disk microscope.

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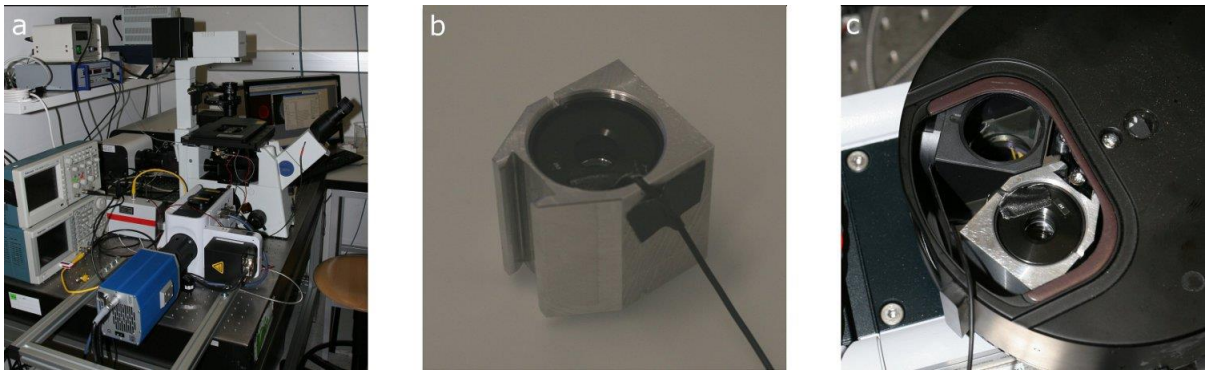

Figure 11: Implementation of ETL and OL in a confocal microscope. (a) The spinning disk unit is attached to the left side-port of the microscope stand. The blue CCD camera is used for imaging. The current converter driving the ETL is controlled by a function generator. The ETL/OL assembly is mounted in a custom filter cube (b) and inserted into the filter turret (c). The filter turret is reinserted into the microscope stand just below the objective turret.

Results

In combination with a 40x NA 1.3 objective (Olympus UPLFLN 40XO), a z-range of 60 micron was possible. Single slices of a z-stack of a pollen grain (100 micron diameter) are shown in Figure 12.

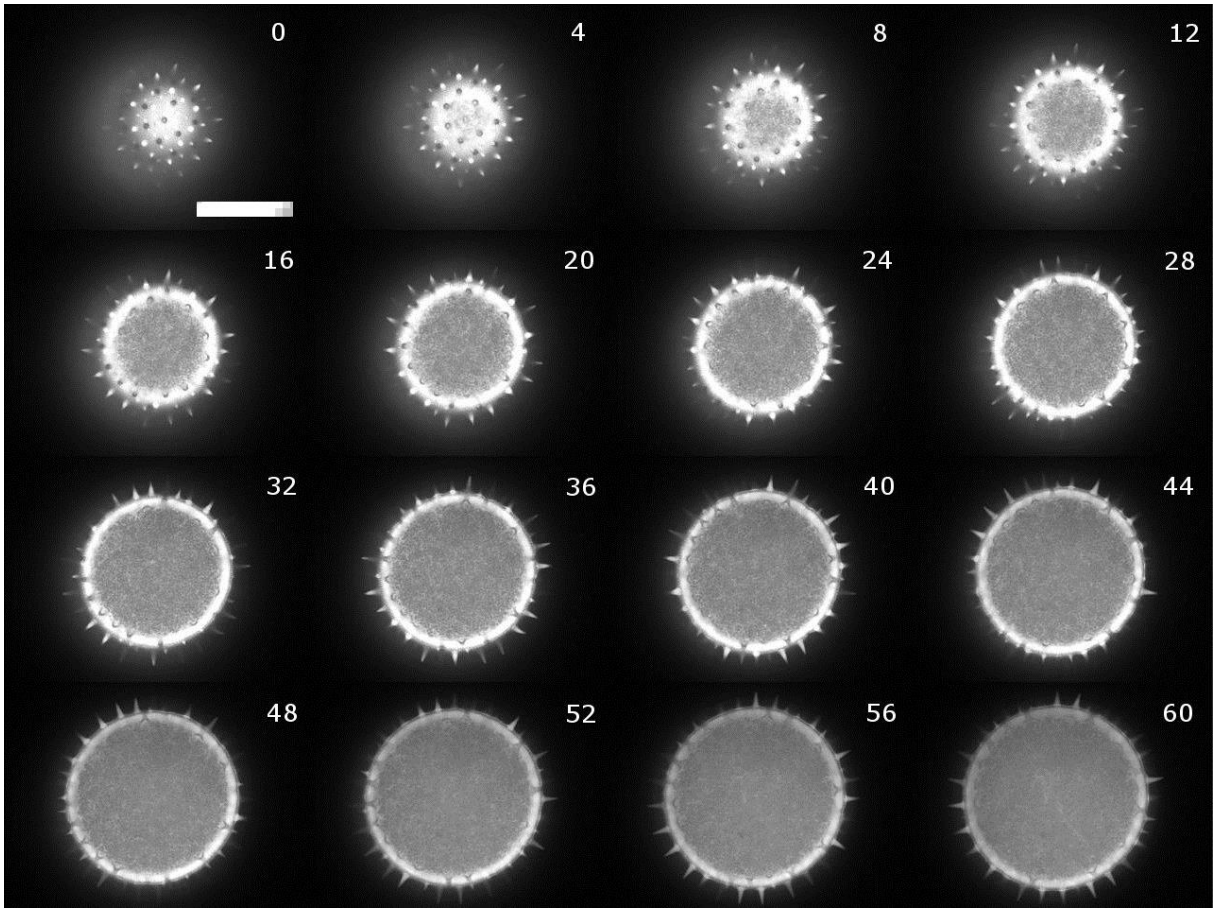

Figure 12: Z-stack of a pollen grain (100 micron diameter) covering a range of 60 micron. The z-positions relative to the first image are indicated. Scale bar: 50 micron. The stack is available as a movie on http://www.optotune.com

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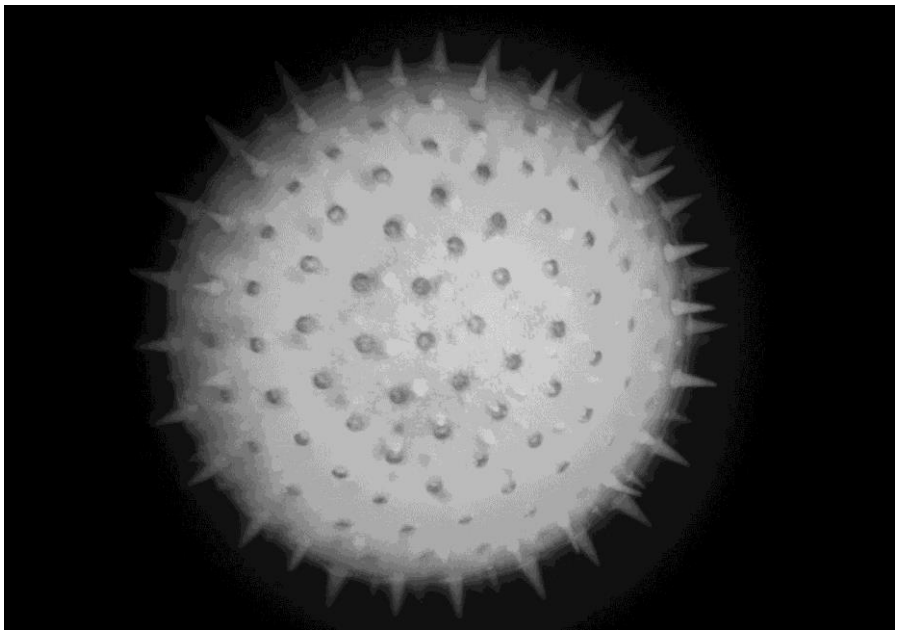

Figure 13: Maximum intensity projection of the data shown in Figure 9.

Application example: Two-photon microscopy

Due to its excellent imaging capabilities in scattering media, two-photon excitation is a technique well suited for fluorescence imaging deep inside tissue (Please see http://www.olympusmicro.com/primer/techniques/fluorescence/multiphoton/multiphotonintro.html for a description of the two-photon method).). Combined with functional indicators of neuronal activity and *in vivo* imaging protocols, two-photon microscopy is a standard method to record population activity from tenths to hundreds of neurons deep inside the living mouse brain. Neurons are distributed across a volume and sampling a single focal plane provides only a hint to the entire activity occurring in local networks. Therefore, fast and simple 3D microscopy techniques are desired – and the use of ETLs provides a very simple and straight forward method. In fact, ETLs and two-photon-microscopy are an ideal combination, due to the following aspects:

- In most two-photon microscopes, axial scanning can be achieved by implementing optical focusing schemes in the excitation path only. This is due to the nonlinear excitation process employed in two-photon microscopy, which can excite fluorophores only at the focus. Moving the focus axially and laterally by modifying the excitation beam is sufficient for 3D two-photon laser scanning microscopy.

- The excitation laser operates at NIR wavelengths, in our microscope at 850 nm. The long wavelength allows wavefront aberrations to be tolerated that would degrade the image severely at visible wavelengths.

- The small spectral bandwidth (10 nm FWHM) of the excitation laser limits the influence of chromatic aberrations that can be introduced by the non-achromatic ETL/OL combination.

- For most biological applications measuring functional cell activity, the change in FOV size introduced by the ETL/OL assembly mounted close to the objective does not interfere with the measurements. To record functional data, the laser has to be pointed at the same set of cells repeatedly over time. If the cells are further apart due to the change in magnification, the respective points are selected to be scanned.

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Setup

The setup used in combination with a custom two-photon microscope is shown in Figure 14.

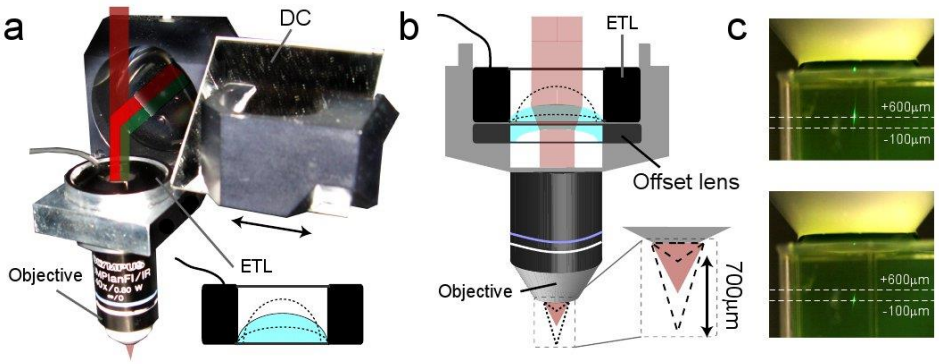

Figure 14: Implementation of the ETL/OL assembly in a two-photon microscope. (a) The ETL and OL are mounted in a custom objective holder which is attached to the detection system of the microscope. Using the movable dichroic DC, emitted fluorescence can be directed to the detection system in the background. An RMS thread is located at the bottom of the holder to allow an Olympus LUMPlanFI/IR 40x NA 0.8 water-immersion objective to be attached. (b) Cut-away sketch of the ETL and OL mounted inside the custom holder. (c) Direct visualization of the two-photon excitation focus in a Fluorescein solution. The focus (green elongated spot) can be moved in a range of up to 700 micron in combination with the 40x-objective.

Results

By tuning the focal length of the ETL between 50 and 200 mm, an axial focusing of up to 700 micron was achieved. The resulting change of the working distance (nominally 3.3 mm) of the objective was 2.8 to 3.41 mm accordingly. For most imaging applications, a much smaller range is sufficient.

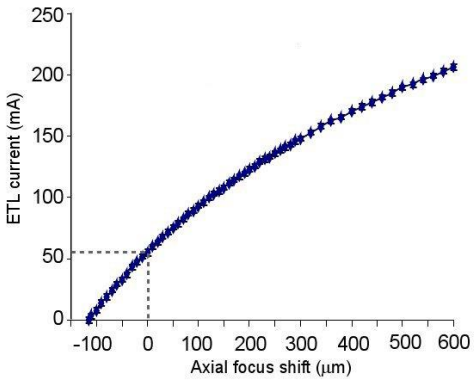

Figure 15: Dependence of the axial focus shift (40x objective, -100 mm offset lens) on the control current applied to the ETL. This set of calibration values was used as a lookup table to focus to a desired position.

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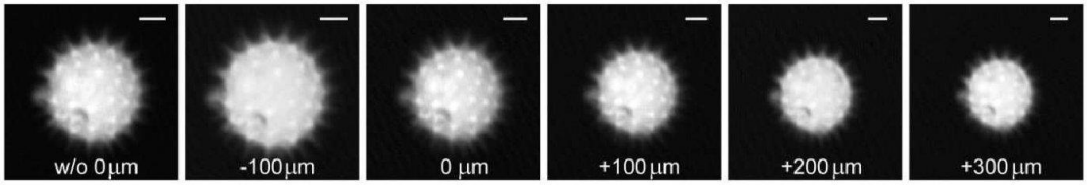


Figure 16: Images of a pollen grain imaged without (left panel) and with ETL-mediated refocusing at varying axial focus displacements, measured with the motorized z-stage of the microscope. Please note the change in magnification / FOV size. Scale bar: 5 micron.

For fast functional measurements of neuronal activity that have been acquired with this setup, please see:

Benjamin F. Grewe et al.: **Fast two-layer two-photon imaging of neuronal cell populations using an electrically tunable lens,** Biomedical Optics Express, Vol. 2, Issue 7, pp. 2035-2046 (2011), doi:10.1364/BOE.2.002035 (http://www.opticsinfobase.org/boe/abstract.cfm?uri=boe-2-7-2035)

Application example: Inspection microscope

In an inspection microscope using standard lens components, the tunable lens is typically placed between infinity corrected objective lens and a tube lens. The following example outlines an off-the-shelf system combining the EL-10-30-Ci with Optem micro-inspection lenses by Qioptiq. All parts offer C-mount threads and thus fit together perfectly. The table below outlines the Z-ranges and resolution achieved.

C-mount camera
½" 5MP sensor

1.5x mini tube lens
P/N 29-90-28-000

Optotune lens
EL-10-30-Ci-VIS-LD-MV

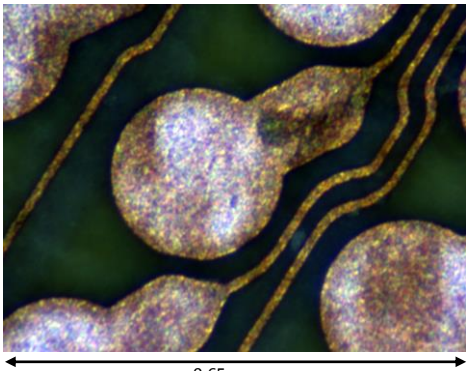
Optem 70XL zoom (0.75x-5.25x)
P/N 399510-309

Coaxial lighting unit with lens
P/N 296515-310

LED ring light (used instead)

Working distance: ~90mm

Optem® is a registered trademark of Qioptiq, Inc



Magnification	1.1x	3.5x	7.9x
Z range	400mm	40mm	8mm
Z resolution	100µm	10µm	2µm
DOF (approx.)	1mm	0.3mm	0.1mm
HFOV	4.5mm	1.4mm	0.65mm

Figure 17: Example of a high-magnification inspection system with off-the-shelf components. The image shows a PCB at 7.9x magnification (track width is 10 µm)

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Using ETLs in microscopy: Checklist

- **Where to insert the ETL/OL assembly in the beam path? Is this position accessible?**
- **Is it possible to position the ETL/OL assembly vertically to the ground within the optical pathway, to avoid any asymmetrical shaping of the ETL surface due to gravity?**
- I**s the beam small enough to match the 10 mm aperture of the EL-10-30?** (This applies to the size of the conjugated pupil as well.) If electrical focusing is not necessary, please consider the use of Optotune's manually tunable lens ML-20-35, which has 20 mm free aperture.
- **Which focusing range do you need to achieve?** In most cases, optical focusing with ETLs needs to be supplemented with mechanical focusing devices to achieve the range needed. ETLs are well suited for focusing ranges from tenths to hundreds of micron with medium- to high-NA objectives. With low-magnification low-NA objectives, a range of 1 mm appears to be possible.
- **Which optical performance requirements have to be maintained?** For example, in two-photon microscopy, the quasi-monochromatic near-infrared excitation light eases the requirements on the correction of chromatic aberrations and other aberrations, yielding a focusing range of hundreds of microns which is needed for in-vivo imaging in the living brain. On the other hand, typical confocal microscopes have much higher demands in terms of optical correction but the typical specimen size is much smaller as well (tenths of microns), which allows successful application of ETLs for certain applications. An optical simulation of the entire microscope usually allows to tailor ETL mediated focusing to necessary requirements.
- **Which focusing speeds are targeted?** Fast focusing with the EL10-30 has been demonstrated (A focus shift of 100 microns in 15 ms with a 40x NA 0.8 objective, corresponding to a change in focal length from 108 mm to 82 mm).

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References and Acknowledgements

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Publications using Optotune's lenses

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