Stage leveling using X-Cite in reflection

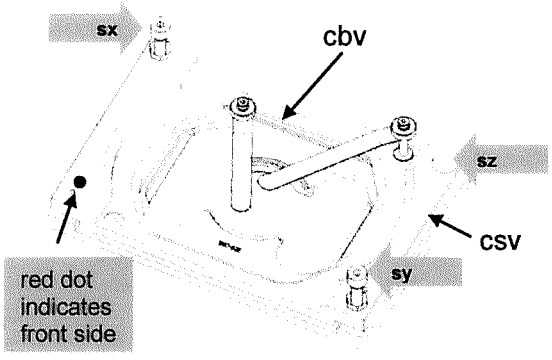

Fig. 1 **Adjustable sample holder – schematic view**

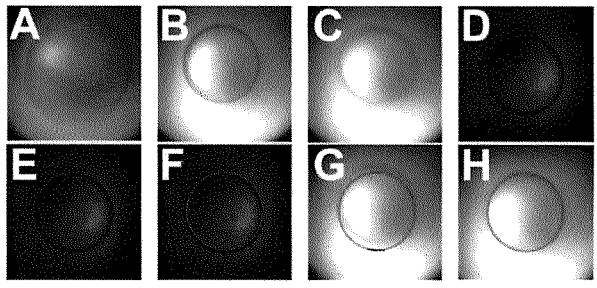

Fig. 2 **Focus control in reflection**

Caution! Adjustable stage insert screws may intersect with parts of the microscope, e.g. the condenser unit. When moving a motorized stage, avoid any collisions. Do not install inserts while the stage is moving. Be careful when switching the condenser front lens.

1. Assemble a fluorescent sample you want to level with sample bottom downwards into the sample holder. The sample site should be clean and free of any immersion oil. Position the sample such that the objective lens is in the middle of the sample. Use WF illumination with the X-Cite at 12% and filter for excitation with the 561 nm line (optimal would be a small band pass to avoid too much light). Swing nosepiece to empty position (make sure no cover is in the empty position) and use preferentially Andor SIM camera, albeit the Andor PALM camera would also work. Take Optovar 1.0 and set EM gain to zero.
2. Draw fiber plug of X-Cite completely out from the X-Cite casing. Start acquisition. The image will be dark since fiber was pulled out. Put fiber back and slowly move in until you will see a coarsely adjusted image to have sufficiently reduced light levels you can work with (Fig. 2A). (Note: with the fiber all in, the lamp is simply to bright leading to oversaturation of the camera). If the illumination is far from being centric, the sample is already strongly tilted and it is recommended to do a pre-adjustment. To this end adjust the edges of the clefts viewed from the right side (Fig. 1, **csv**) and back side (Fig. 1, **cbv**) to be parallel using screws **sy** and **sx**, respectively (Fig. 1). The cleft's width should be approximately 2 mm as preset by the factory, but can be adjusted with screw **sz** if needed.
3. Now pull (or push) fiber on the ELYRA site until the dark ring, which is already faintly visible in its contours, appears focused (Fig. 2B). Dependent on the sample the focus could look worse, especially when the reflecting planes are wavy or you get reflection from different planes (Fig. 2C).
4. Move in 10 x objective and focus the already visible ring with the z-drive of the stand. The ring appears now well focused as long as the sample is not completely unsuitable (Fig. 2D)
5. Mark the Ring with a ROI from the **Graphics** tool (3 point-circles will work best) (Fig. 2E).
6. Mirror the circle on the center of the image. (Note: If the circle is already centered in the middle that step is not required; deviations <20 pixel SIM camera or 10 pixel Palm camera are no problem and can be tolerated.). To mirror the coordinates of the ROI **X, Y** (visible in the **Graphics** tab), calculate the new values as follows: X' = Xmax-X & Y' = Ymax-Y with Xmax and Ymax representing the number of pixels of the camera in X and Y-direction. E.g. if the coordinates X and Y would have been 502 and 512, respectively, the new coordinates X' and Y' would be calculated using the SIM camera to 1004-502=502 and 1002-512=490.
7. Draw a new circle with the same diameter in the original image with the new coordinates (Fig. 2F). The old circle is not necessary any more and can be switched off or deleted (Fig. 2G).
8. Turn nosepiece back to free position. Than level stage by turning the screws of the sample holder (Fig. 1, **sx** & **sy**) until the more or less focused circle of the fiber overlaps with the ROI-circle (Fig. 2H).