

# XM-2 Operating Manual

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## Safety is our highest priority

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Safety is paramount while you are working at the NCXT. Consequently, all users must complete appropriate safety training courses before they can gain access to the facility. Contact NCXT staff to determine your current training status and the courses you must take, either before coming to the facility or on your arrival. Note: Although you may have completed training courses for earlier visits, some courses need to be retaken every year. Please check your training status before you arrive.

Once you arrive at the ALS an experimental user safety sheet must be signed prior to the start of every data collection run on the x-ray microscope. The experiment safety sheet documents which is using the facility at any given time, and ensures that everyone who takes part in an experiment is correctly certified.

Not following safety instructions will almost certainly result in the termination of an experiment. Tampering with safety devices, or otherwise trying to circumvent safety procedures is a very serious matter.

**NCXT and ALS staff are here to help you accomplish your experimental goals, and work in complete safety. If you have any doubts or questions about your work, or the work of those around you, please contact a staff member immediately.**

Emergency services – paramedic, fire and security – are located adjacent to the ALS. They can be contacted anytime night or day. Please don't hesitate to call for assistance in case of fire, illness or any other emergency. If in doubt, call anyway.

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**In an Emergency: Dial 7-911 from any lab phone or 911 from your cell phone**

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Liquid nitrogen can be obtained for the cold traps and cryostage from the 50 liter vessel located at the door to the x-ray microscope or, in the event that this supply runs out, from the large Dewar directly across the walkway from the beamline. **BE SURE TO ALWAYS USE PERSONAL PROTECTION EQUIPMENT WHEN HANDLING CRYOGENS INCLUDING FACE MASK AND GLOVES.**

While we are on the subject of safety, please no eating or drinking in the experimental areas. In addition to putting yourself at risk of ingesting toxic chemicals, spilled food or liquids can cause expensive damage to sensitive instruments and equipment, or cause contamination. Please, resist the temptation to snack while you work.

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# INTRODUCTION

The National Center for X-ray tomography (NCXT) soft x-ray microscope is located in sector 2 of the Advanced Light Source. Currently, this is the only beamline operating in this sector of the synchrotron, and therefore should be relatively easy to find. The specimen preparation laboratories are located on the 2nd floor of building 80 in rooms 80-210, 80-203 and 80-208.

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## XM-2: BACKGROUND AND BASICS

Beamline 2.1 (XM-2) is a transmission soft x-ray microscope in sector 2 of the Advanced Light Source at Lawrence Berkeley National Laboratory. XM-2 was designed, built and is now operated by the National Center for X-ray Tomography (NCXT) as a National Institutes of Health Biomedical Technology Research Resource. XM-2 is equipped with a cryogenic rotation stage to enable tomographic data collection from cryopreserved cells, including large mammalian cells. During data collection the specimen is illuminated with 'water window' x-rays (284-543 eV). Illuminating photons are attenuated an order of magnitude more strongly by biomolecules than by water. Consequently, differences in molecular composition generate quantitative contrast in images of the specimen. Soft x-ray tomographic microscopy is an information-rich 3dimensional imaging method that can be applied either as a standalone technique or as a component modality in correlative imaging studies.

An overview of XM-2 instrumental layout is shown in Figures 1A and B, and the operating parameters summarized in Table 1. The complete beamline - from the bending magnet source to the CCD detector - is 19.71 m long. Currently the microscope can operate between 400 and 1300 eV. To date all experimental data have been collected on XM-2 using 'water window' illumination (284-543 eV), however in the future it is conceivable that data collection at higher energies may be desirable. To accommodate higher energy modes of operation, all microscope components from the mirror chamber downstream to the CCD detector are mounted on plates supported by air pads. When activated the pads levitate the entire beamline on a frictionless cushion of air. Consequently, the entrance and exit angles of the x-ray source can be adjusted easily from 0.5° to 3° with respect to the mirror surface (Figure 1B). This adjustability allows the operational energy range of the microscope to be extended up to 4 keV. Achieving this operational mode only requires simple upgrades to the radiation shielding, rather than requiring a major beamline redesign. This upgrade will be implemented once there is sufficient demand and scientific justification.

The optical setup of XM-2 is that of a classical two-lens bright-field light microscope, and uses Fresnel zone plates as both condenser and objective lenses (Attwood, 1999) (Kirz *et al.*, 1995) (Falcone *et al.*, 2011). The flat mirror acts as a cutoff filter for high-energy photons. The condenser lens creates a 40x-demagnified image of the x-ray source in the specimen region. Subsequently, the objective lens transmits a magnified image of the illuminated specimen onto the CCD detector. In combination with a central beam stop and a pinhole in close vicinity to the specimen, the condenser zone plate (as a diffractive element) acts as a linear zone plate monochromator (Attwood, 1999).

A low field (1.3 Tesla) bend magnet source at the ALS is ideal for soft x-ray microscopy as the peak brightness occurs over the important 'water window' wavelength range. The ALS storage ring operates at 1.9 GeV and a constant beam current of 500 mA. The source is sufficiently stable that flat field images are acquired only at the start and end of each tomographic data set, rather than after each individual projection image. At bend magnet 2.1, a recent upgrade to the ALS lattice resulted in the electron beam being 32.7mm wide and 32.6 mm high. It is a line source with the vertical divergence controlled largely by the synchrotron cone from 1.9GeV electrons traversing a 1.3T bend magnet. At a source to condenser distance of 18.2 m, a 1-cm diameter condenser zone plate is optimally filled with 'water window' photons in the vertical direction and captures 0.5 milliradians of the horizontal line source. The source is de-magnified 40 times onto the image plane and the modest optical performance of the condenser lens produces a 1x1 mm spot. The condenser is scanned using a precision X-Y scanner stage to produce a flat-field illumination pattern that fully utilizes the dynamic range of the detector when imaging cylindrical specimens. Since the condenser aperture is highly overfilled the specimen illumination remains stable over time. Optimizing the alignment of XM-2 is straightforward, and has proven robust to source relocations due to synchrotron upgrades and seismic disturbances. This factor has contributed significantly to the large number of whole cell tomograms generated by Users of the facility.

## **Front-end Assembly**

The 'front-end' assembly interfaces the end-station with the storage ring. The front-end consists of a number of ultra-high vacuum fast valves interlocked to pressure sensors along the beamline. The fast valves rapidly isolate the storage ring in the event of an accidental vacuum breach further along the beamline. The front-end also contains a number of fast water-cooled photon stops, apertures and radiation shields.

## **Branchline**

The ALS bend magnet that provides illumination to Beamline 2.1 produces a beam with a horizontal swath of 200 milliradians and a vertical emittance on the order of 3 milliradians for x-rays at energies appropriate for soft x-ray microscopy. A beam-defining aperture in the front-end generates a beam of 2 milliradians in both the horizontal and vertical direction. The total power generated from the bend magnet source is 3 KW into the full 200milliradian horizontal emission pattern. After collimation by the front-end apertures, the total power leaving the concrete shielding of the storage ring is ~30 Watts. The spectrum of radiation allowed into the beamline extends from the infrared range and up to the energy of the stored electrons, in this case 1.9 GeV. The very high-energy photons are generated from bremsstrahlung processes due to electrons in the storage ring interacting with residual gas molecules in the storage ring vacuum chamber. This results in a spectrum of gas bremsstrahlung photons with a peak flux at approximately 10 MeV. The Beamline 2.1 branchline modifies the spectrum of the radiation and controls the beam direction. The branchline also stops high-energy bremsstrahlung radiation by using a 30 cm cube of lead in line with a direct path to the source. A flat x-ray mirror deflects bend-magnet radiation and the spectrum of deflected x-rays depends on the grazing angle of the mirror and the coating used. For a 3° grazing angle and a nickel coating, x-rays above 700 eV are strongly attenuated; therefore this choice of angle and coating is most suitable for water window imaging. Another function of the branchline is to provide another level of vacuum protection for the storage ring via an additional fast shut-off valve.

## **X-ray Mirror**

The x-ray mirror is located just outside of the ALS shield wall. This optic redirects x-rays with the desired spectral range to the condenser system of the microscope. In addition to this filtering action, the mirror is also fully motorized to allow alignment of the illumination beam with the optical axes of the microscope end-station. The mirror is a flat optic with a measured tangential slope error of 1.14  $\mu$ rad RMS over 700 mm, corresponding to 67 km radius of curvature; and a sagittal

slope error of 7.85  $\mu\text{rad}$  RMS over 40 mm, corresponding to 33.38 km radius of curvature. The surface roughness was 2.5A RMS. We selected a silicon substrate 700 mm in length and 40 mm wide with two reflective stripes: one nickel and the other gold. The extra gold coating was chosen to enable the microscope to work at higher energies as well as lower energies not reflected by the nickel mirror, in particular energies close to nickel x-ray absorption edges. The mirror (SESO Inc., France) is water-cooled via a nickel coated cold finger immersed in a liquid gallium contact bath. The complete mirror box assembly is a UHV vacuum system held at low pressure by a 500  $\text{ls}^{-1}$  ion pump. The mirror box, associated motorization and LabView ([www.ni.com](http://www.ni.com)) control system was provided by Oxford Danfysik ([www.oxford-danfysik.com](http://www.oxford-danfysik.com)) according to specifications from NCXT staff.

## Delay Line

This section of the branchline is a simple but necessary part of the vacuum isolation system. To exploit the wide divergence angle of a bend magnet x-ray source, and to keep the first mirror a simple planar optic, it is necessary to place the x-ray microscope close to the storage ring. Therefore, a vacuum delay line is required for storage ring vacuum protection. The delay line consists of two large vacuum tanks connected by a relatively low pumping conductance tube. Each vacuum vessel is independently pumped. The chamber closest to the storage ring is connected to an ion pump. A turbo pump maintains vacuum in the other chamber. There are also vacuum isolation valves between the chambers, as well as a high-speed fast valve that acts to quickly isolate the mirror chamber during a vacuum failure. Such a failure could result from the rupture of one of the thin, 100 nm thick silicon nitride windows in the downstream components of the x-ray microscope. The large volume of the delay line chambers would attenuate such a pressure spike, slowing the pressure rise in the ultra-high vacuum regions of the beamline. This would allow time for the fast valve mechanisms to close and prevents the pressure in the mirror box from rising to a level too high for the mirror box ion pump. All valves are controlled by a computerized equipment protection system called the "Equipment Protection System," otherwise known as EPS.

## X-ray Illumination System (KZP box)

The x-ray illumination system is analogous to the condenser system used in bright field visible light microscopy. It focuses the source radiation onto the specimen, optimally matching the numerical aperture of the specimen illumination to that of the objective micro zone plate lens. The condenser zone plate (known as KZP to conform to the original German nomenclature) has 41667 zones made with approximately 200 nm thick nickel. The diameter of the optic is 1 cm with a 5 mm central stop. The condenser zone plate lens also functions as an x-ray monochromator. The bend magnet source radiation is broadband, but transmission x-ray microscopy requires narrow-band illumination due to strong chromatic effects in zone plate imaging. To circumvent this problem, the condenser is configured as a linear monochromator, formed by the condenser zone plate and a pinhole placed close to the specimen (Hirai *et al.*, 1999). The relative bandwidth of this monochromator is determined by the width of the condenser zone plate lens, the size of the pinhole and the source demagnification factor;  $(E/\Delta E)$  is a value of 300 for the lens and pinholes typically used. The focal length of a Fresnel zone plate lens is inversely proportional to the wavelength focused. Consequently, moving the condenser zone plate relative to the pinhole can vary the photon energy used to illuminate the specimen.

The total condenser zone plate travel is 1600 mm, with a minimum condenser to specimen distance of 37 mm. The corresponding imaging wavelength therefore varies from  $1 \text{ nm} \leq \lambda \leq 13 \text{ nm}$ . Both the condenser and the condenser pinhole are mounted on a 3-axis, computer-controlled stage to allow alignment of all components to a common optical axis.

The bend-magnet x-ray source is imaged through the monochromator pinhole and onto the specimen by the condenser zone plate. This type of illumination is referred to as critical illumination. The spot size of the imaged source at the specimen is less than  $2\mu\text{m}$  in diameter; as a result, it is necessary to scan the condenser in order to uniformly illuminate the

desired field of view. The field of view for Beamline 2.1 is  $15\mu\text{m} \times 15\mu\text{m}$ . The condenser is mounted on a precision-encoded piezoelectric flexure stage and the scanning pattern is adjustable and synchronized with image acquisition on the CCD. The stage can accommodate multiple condenser lenses to allow optimization of zone plate lifetime. As one condenser degrades due to radiation damage, a new condenser can be mounted, allowing the old condenser to be used for alignment and non-critical imaging procedures. The condenser is monitored for signs of degradation by observing the size and shape of the reimaged source, and recording a spectral image of a standard titanium oxide specimen. That said, minimal degradation of condenser performance has been observed over time. KZP optics that have been in heavy use for the past four years are still capable of high quality imaging.

When the 'best quality' image is required, the high performance condenser is moved into position for imaging. All stages in the condenser box are indexed and position-encoded to a resolution better than 100 nm to enable simple setup and alignment prior to each imaging session. The condenser vacuum box is pumped by a turbo-molecular pump as well as a liquid nitrogen cooled cryo-trap. The cryo-trap ensures a clean vacuum, which helps to prevent x-ray induced contamination build up on the x-ray optics. Once the KZP chamber has been pumped to a low pressure, the turbo-molecular pump can be switched off and maintained at low pressure by a cryo-trap and vacuum pumps in the branchline. This helps stop vibration from being transferred to the cryo-rotation stage, which could lead to degradation of the x-ray image. A neutral density filter wheel, at the entry of the KZP box, can be used to fine tune the acquisition protocol and avoid over-exposure or saturation of the CCD detector during binned video mode operation. This mode enables automation of the specimen alignment procedures without the introduction of excessive radiation damage.

### **X-ray Image Formation System (MZP box)**

The micro zone plate (MZP) function is analogous to the objective lens in bright field light microscopy. Namely, it gathers transmitted light from the specimen and focuses a magnified image of the specimen onto the CCD detector. The MZP is housed in a vacuum enclosure containing two precision encoded 3-axis (X, Y, Z) stages. One stage controls the position of the MZP; the other stage positions a phase ring in the MZP's back focal plane. As the energy or magnification used for imaging varies, both the MZP and phase plate need to be adjusted relative to the specimen. For direct absorption imaging the phase plate is moved out of the beam path completely. This is the only fully operational mode of imaging for synchrotron Users at the present time.

The vacuum requirements in this part of the microscope are not as strict as those for the KZP box and other parts of the instrument closer to the storage ring. However, it is important to ensure a low hydrocarbon environment to prevent x-ray induced polymerization of organic compounds on the zone plate optic or other sites of high x-ray flux, such as the thin window on the vacuum isolation cone. The microscope can accommodate two or more objective lenses, allowing multi-resolution imaging of a specimen. The MZP support arm is attached to a kinematic base and thus enables the whole structure to be easily removed through the vacuum hatch at the top of the MZP box. To accommodate different imaging conditions, such as resolution, working distance, field of view and depth of field (DOF) several designs of MZPs were fabricated using electron beam lithography. The MZPs are all made with nickel zones and have a diameter in the region of  $70\mu\text{m}$ . The higher resolution MZP has an outer zone width of 50 nm, a focal length of 1.5 mm and a DOF of  $\pm 2\mu\text{m}$  at a wavelength of 2.4 nm. The larger DOF MZPs have an outer zone width of 80 nm, a focal length of 2.4 mm and a DOF of  $\pm 5\mu\text{m}$  at 2.4 nm wavelength. These zone plate parameters were chosen to maximize the types of specimen that can be imaged by SXT. A planned future development is to increase the imaging resolution on XM-2. In 2-D imaging, state of the art zone plates have demonstrated to deliver spatial resolution in the regime of 10 nm (Chao et al., 2012, Vila-Comamala, 2009, Rehbein et al., 2012). Since the DOF decreases quadratically with resolution, using high resolution MZP optics will require techniques such as deconvolution be applied when imaging specimens thicker than the DOF. Alternatively, use of

non-conventional optics, such as cubic zone plates, could potentially increase the resolution without such a significant decrease in DOF.

## Detector

The detector of choice for soft x-ray imaging is currently a back-thinned, cooled CCD. The CCD camera on Beamline 2.1 was manufactured by ANDOR instruments ([www.andor.com](http://www.andor.com)) model iKon-L DO936N BN9KH. The camera has 2048 x 2048 pixels, each of which is 13.5  $\mu\text{m}$  x 13.5  $\mu\text{m}$ . The CCD is back-illuminated with no anti-reflection coating and has a 16bit, 1 MHz readout analog-to-digital converter (ADC) and 14bit, 3-5 MHz readout capability. The CCD chip in this camera is manufactured by E2V Inc. ([www.e2v.com](http://www.e2v.com)), and is cooled to  $-30^{\circ}\text{C}$  by a Peltier cooling stack. As the microscope magnification is changed, the camera needs to move along the optical bench; it has a total range of motion of 200 cm. In addition to direct cooling of the CCD to reduce dark current noise, a liquid nitrogen cooled water trap is connected to the CCD vacuum system. This ensures that any moisture present in the vacuum system preferentially condenses and freezes in the cold trap rather than on the surface of the CCD. A fast shut-off valve is also placed just in front of the camera; this valve activates in the event of a rupture of the thin MZP window, again protecting the detector from atmospheric moisture.

## Cryogenic Specimen Rotation Stage for Tomographic Data Acquisition

NCXT staff designed and constructed a cryogenic specimen stage (cryo-stage) that can be used for single axis tomographic measurements on both flat and cylindrical specimens. For specimens loaded into thin-wall glass capillaries, acquisition of tomographic data sets that take advantage of  $360^{\circ}$  of rotation is typical. For extended flat specimens full rotation is not possible due to both the mechanical interference between the specimen and microscope components (Figure 2A), and the thickness of a flat specimen increasing as a function of increasing tilt. The system is designed to allow up to  $\pm 75^{\circ}$  tomography on such flat specimens. Rotation of the specimen is provided around an axis defined by two high precision spheres in the rotation stage. A tip/tilt eucentric correction stage rotates with the specimen to bring the region of interest onto the rotation axis (Figure 2B).

The cryo-stage is based on many of the ideas and techniques commonly used in macromolecular crystallography. The specimen is mounted on an X,Y,Z- $\theta$  stage, and an encoded motorized stage is used to center the region of interest in the specimen on the rotation axis. A two-axis, picomotor-actuated stage is positioned above the specimen tube and rotates with the specimen. Centering for specimens mounted in capillaries is first performed using a light microscope to ensure no more than a 1  $\mu\text{m}$  deviation as the specimen is rotated through  $180^{\circ}$ . Further improvement in alignment is performed using a sequence of 4 low-dose x-ray images to first view, and then to position the rotation axis to better than 0.25  $\mu\text{m}$ . The first alignment image is taken at  $0^{\circ}$  rotation and the position of the specimen center is recorded. The specimen is then rotated to  $180^{\circ}$ , the new position is noted, and then the axial alignment stage is used to position the specimen at the mid-point of the  $0^{\circ}/180^{\circ}$  specimen positions. The same procedure is used again for the  $90^{\circ}/270^{\circ}$  specimen positions. After this procedure the specimen is well aligned to the rotation axis of the cryo-stage, and a complete tomographic data set can be collected without any refocusing of the specimen. The entire alignment procedure takes less than 20 seconds and is now being automated. The capillary tubes are of sufficiently high symmetry that 50-100  $\mu\text{m}$  of the tube length is suitable for acquisition of a tomographic projection series after a single axial alignment.

The XM-2 cryo-stage is built around a Dewar filled with liquid nitrogen which provides a cold supply of Nitrogen and Helium gas. These gases are used to keep the specimen area and the specimen itself at cryogenic temperature. The specimen area

is located in an insulated arm extending from the Dewar. A system of heat exchangers and heat shields is used to guarantee efficient cooling and shielding of the specimen.

The specimen is maintained at low temperature and atmospheric pressure by a stream of liquid Nitrogen-cooled Helium gas. To ensure that the minimum flow of gas is used to maintain an adequately low temperature, we used a conduction-cooled intermediate heat shield. This heat shield provides most of the cooling power required to keep components that are close to the specimen cold, including items such as the KZP and MZP cones. The slow flow of cold Helium gas also prevents the ingress of moisture. In Figure 3 we show a simplified diagram of the cryogenic gas flow used to maintain the specimen at low temperature.

The intermediate heat shield is maintained at 90K by thermal connection to a nitrogen gas primary cooling loop. The primary loop also cools a dual heat exchanger system that generates the low temperature helium gas used for direct specimen cooling. This arrangement greatly reduces the flow of helium gas required to keep the specimen cold. The use of a low flow rate for final stage specimen cooling reduces the volume of helium gas exhausted to the atmosphere and helps prevent ice build-up and generation of local thermal gradients that cause the specimen to drift during image acquisition.

Specimen stability is a crucial part of high-resolution x-ray imaging. For a single high spatial resolution TXM image using a synchrotron source, one has to expect an exposure time on the order of a hundred milliseconds. This time scale is long enough for deterioration of the image quality by movement of an unfixed biological specimen in an aqueous environment and by radiation induced damage. Tomographic investigations at a cellular level therefore require sufficient fixation procedures. Cryopreservation has been shown to be an excellent approach, allowing the collection of hundreds of high-resolution x-ray images of biological specimens.

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## **XM-2: OPERATING PROCEDURES**

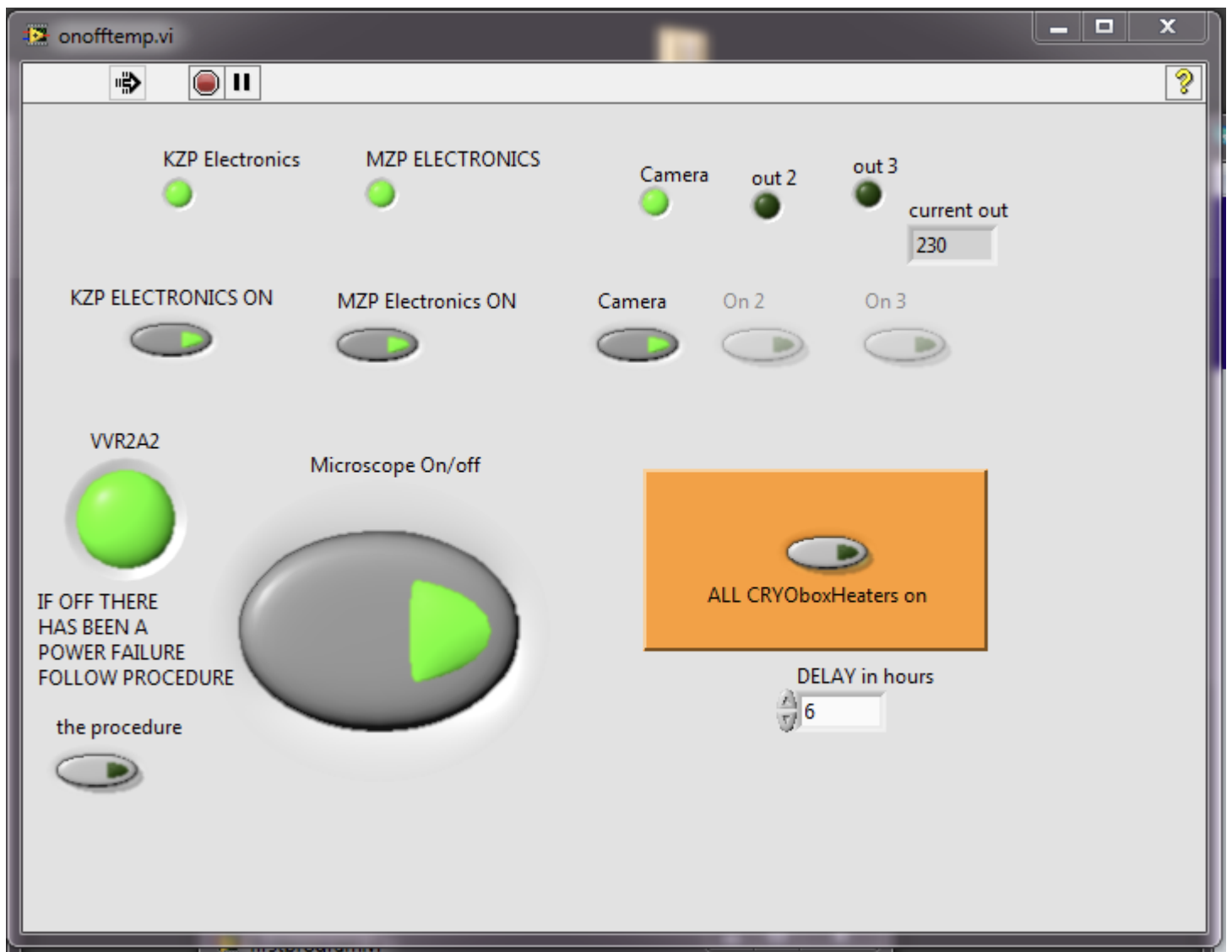
### **Turning on the microscope & initial preparations**

Before launching the graphical user interface first:

1. Turn on the Microscopy

- a) Open labview and choose the “first program”, (click run) and turn the microscope on. It turns on the KZP, MZP and X-ray Camera

- b) The heater should only be switched on when the specimen reservoir is cooled down to keep condensation and ice off the sample stage



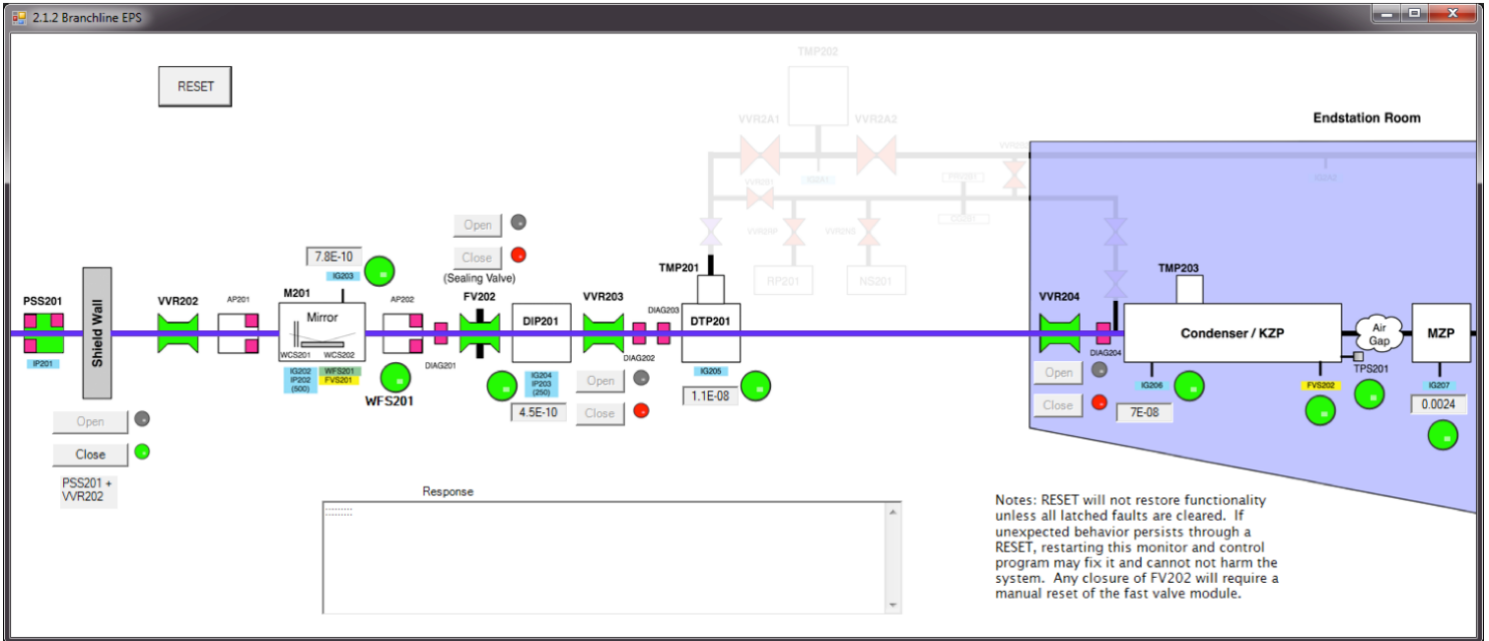
2. Check the beam comes from synchrotron or not

a) Once all necessary hardware has been switched on, open the Branchline Equipment Protective System (EPS) by clicking on the EPS icon found on the SXT computer desktop.

b) Before opening the valves on the branchline, check the pressure gauges on the beamline. If the pressure is not sufficiently low, indicators will be red and you will not be able to open the shutters (In which case, help from Mark Le Gros, or an ALS control room operator is required). Pressure at IG207 should be around  $10^{-5}$ , pressure at IG206 around  $10^{-7}$ , IG205 is around  $10^{-8}$ , IG204 and IG203 are close to  $10^{-10}$ . These pressures will change once the x-ray beam is open, with IG203 and 204 rising to low  $10^{-9}$ .

c) You need to open the valves VVR204 (far right button) and VVR203 in order to open the VVR202 (far left button) to let the beam to come through into the microscope. The black line turns purple when the x-ray beam is present. If after trying to open VVR202, the button is blinking, you need to call "RING" and contact the ALS duty operator (extension 7464). In this situation another shutter needs to be opened by the ALS operators, this is not usually the case but sometimes after a long accelerator shutdown the beamline maybe offline. If the black line does not turn purple after opening all necessary valves, check to insure there is beam via the ALS website. If the synchrotron has beam, but beamline 2.1. does not, please contact a person responsible for the beamline.





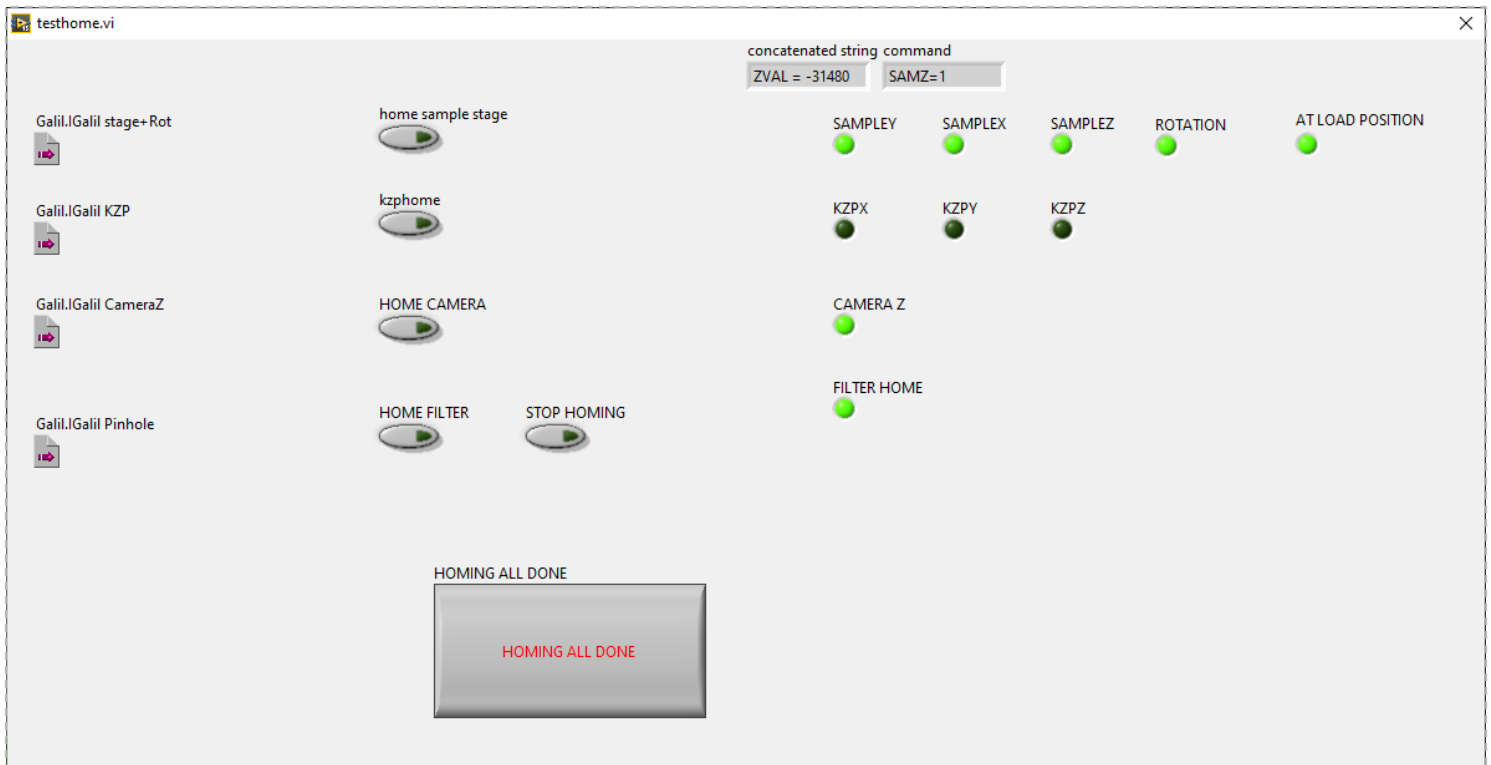
3) Only if the vacuum is okay and the black line turns purple as shown above, continue and turn on the nitrogen and helium gas flow just outside the beamline hutch door. Turn both gas flows on by opening both the nitrogen and helium gas bottles. Let it run for about 10 min before adding liquid nitrogen to the sample stage cryotank.

4) Slowly fill the liquid nitrogen cold traps on the condenser (KZP) box and the micro-zone plate (MZP) box.

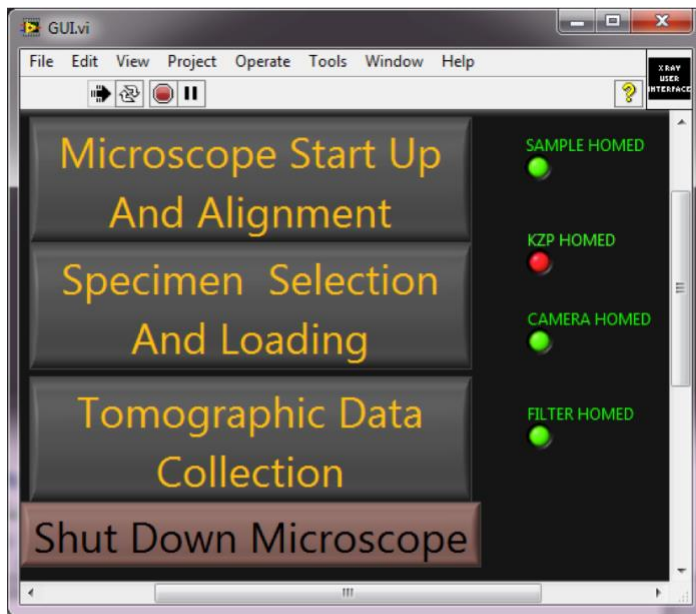
## Initial microscope alignment

1) Open the LabView icon "Axil" on the desktop folder. Click on gui.vi and ignore occurring errors. Execute the program by clicking on the black arrow.

2) The soft x-ray microscope elements use a variety of different motors and encoders, and each element must first be homed to the correct position on starting the microscope. Press "Microscope Startup & Alignment" and then "Home Microscope". Home the sample stage, the filters and the camera, but do **not** home the KZP. Homing takes several minutes. The indicators turn green when homing is complete. Press "Homing all done" when done. The position of the pinhole and the MZP (x and y axis) is encoded by an absolute encoder (LVDT) and does not need to be initialized. The Camera can move on the optical axis but usually does not require movement during normal operation. (See troubleshooting section)



3) Next, align the microscope at room temperature to find, center and optimize the throughput of the beam on the camera chip. Press “Microscope Alignment” and write down the coordinates for the KZP, MZP and pinhole. Move the X-ray camera towards the sample stage by  $-10^6$  um. Make sure the parameters are set as “2X2 binning” and “100% filter transmission”.

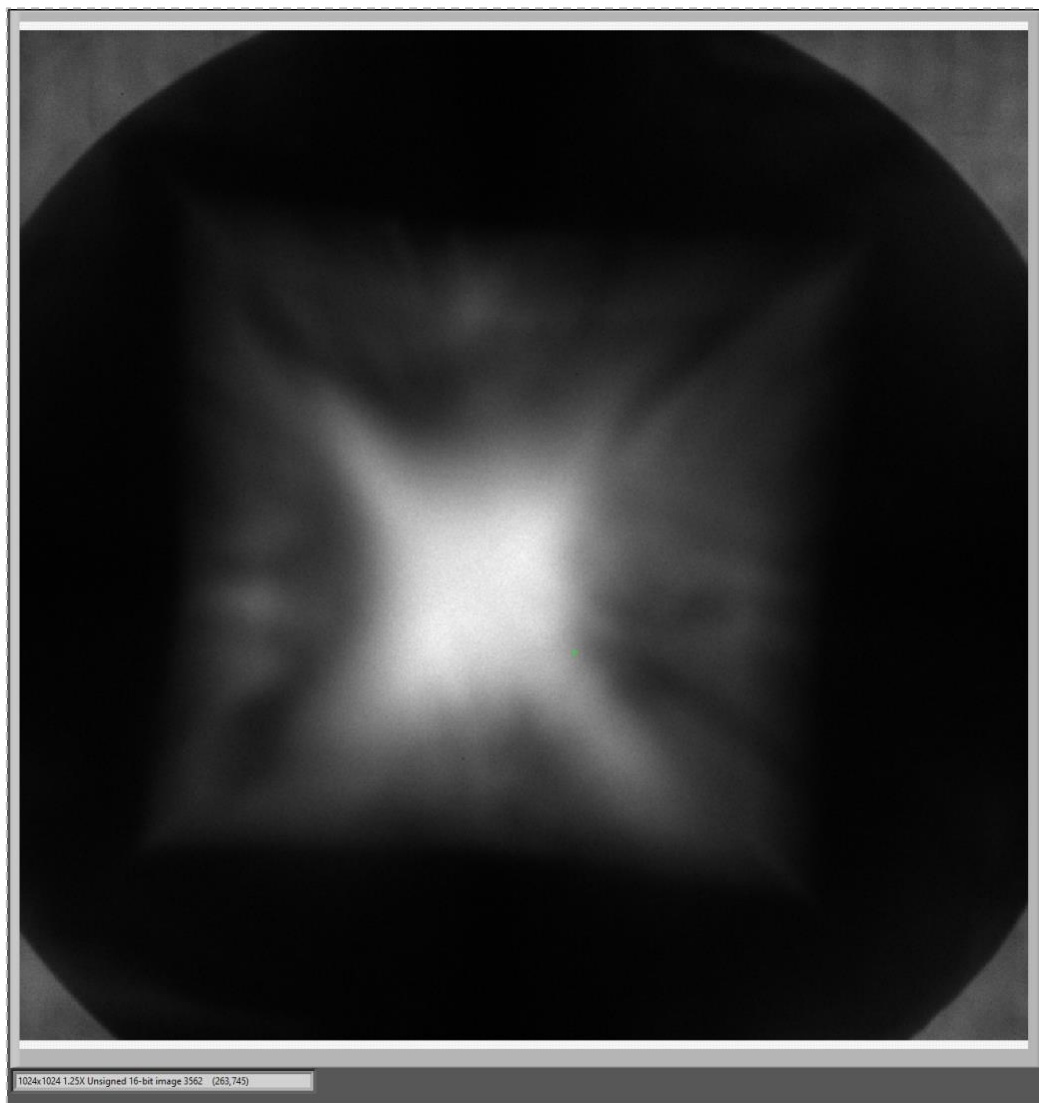


The beam alignment module interface window opens on the right screen. This module gives you access to the optical elements described above. Exposure time can be selected by pressing the “generate pattern button”. Choose the “no scanning” mode for the alignment procedure. The position of the KZP, MZP and pinhole can be read out at "Position" and the

required displacements can be entered in the "Amount" window. Pressing the button "Move X" moves the KZP on the X axis by the X amount entered. Similarly for the Y and Z axis. After choosing the "No Scanning" mode, take a snapshot by pressing the "Snapshot" button.

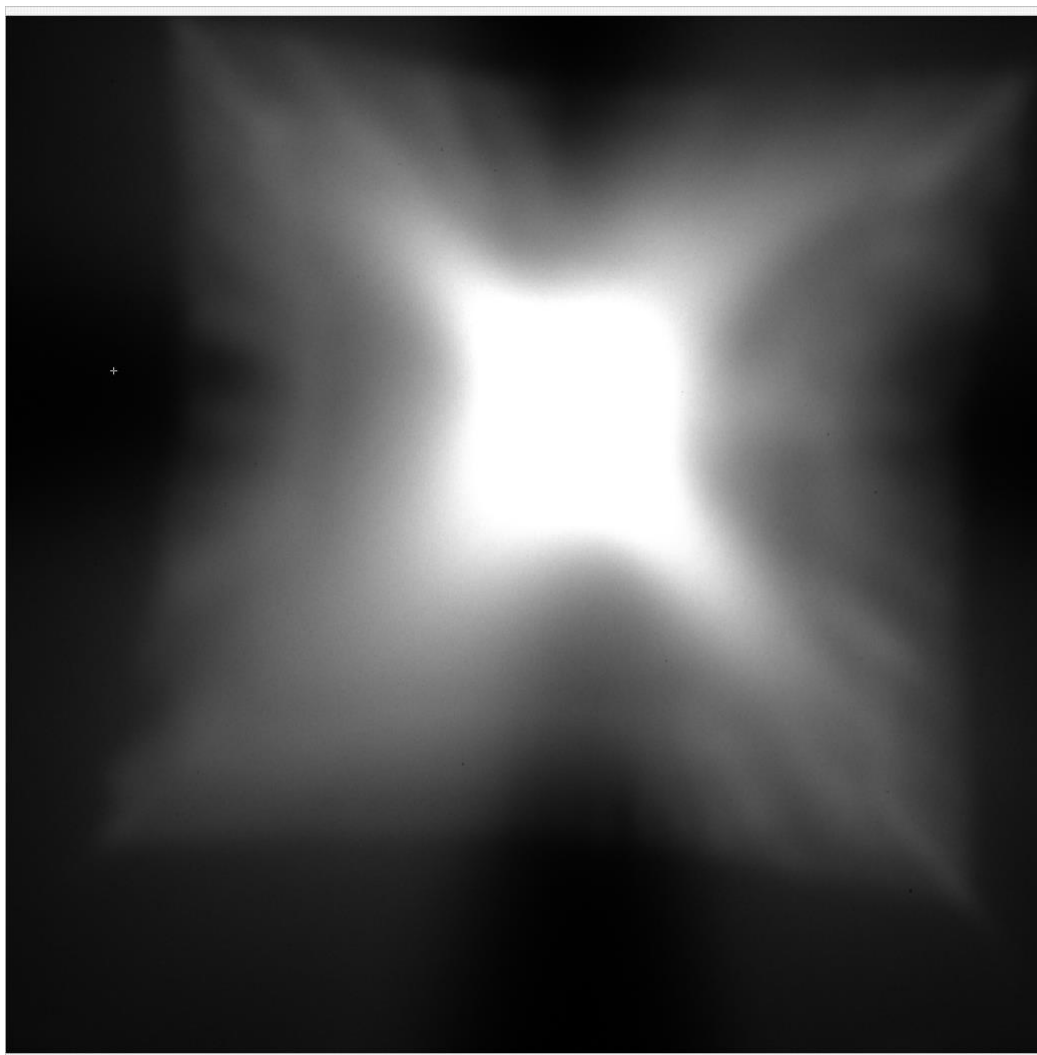
You want to have the entire brightest square in the field of view of the camera. To do this, move the MZP. The maximum displacement for the MZP must not be more than 5 microns. Otherwise you risk losing all light coming through. X axis motion of the image is intuitive (moving the square image to the left requires a negative displacement, moving the square image to the right requires a positive movement). The Y axis is reversed (moving the square image to the top requires a negative displacement. Moving the square to the bottom requires a positive displacement). A guide to the movement direction of the image when the MZP is moved is represented by the diagram on the "Aligning the Beam" GUI. (Summary of this diagram:  $+X = \rightarrow$  ,  $-X = \leftarrow$  ,  $+Y = \downarrow$  ,  $-Y = \uparrow$ ). After you centred the field-of-view square, you need to find and centre the condenser spot to the centre of the square by moving the KZP. Amount for the moves must not be over than 10 microns, 10 to 20 um total movement of the condenser is usually all that is required. For the condenser the X axis motion is reversed and Y axis is intuitive. To summarize the moves of the KZP:  $(+X = \leftarrow$  ,  $-X = \rightarrow$  ,  $+Y = \uparrow$  ,  $-Y = \downarrow$ ).

After several displacements (rarely more than 100 microns on X and Y axis), you should obtain something like this:



## Cooling down the sample stage & re-alignment

- 1) Once the beam is centered, the cooling down procedure can be launched by pressing the “Start Cooling down” button. This will automatically increase the He flow to 0.6 (the far right pressure indicator on the pressure indicator board).
- 2) You need to fill the white polystyrene keg/zeolite tank with liquid nitrogen in order to flush the system. Open the two green valves at the zeolite tank to start the nitrogen flow.
- 3) After 10-15 min, you can increase the pressure of Nitrogen gas to 30 and fill the cryobox with liquid Nitrogen. **You need to turn on the heaters.**
- 4) When the temperature at the specimen reaches below 0 Celsius increase the nitrogen flow to the maximum flow rate.
- 5) The microscope needs to be re-aligned after cooling. Write down the positions for the KZP, MZP and pinhole and re-do the alignment as described in the previous section.



- 6) Drive the camera back to its initial position ( $+10^6$  um) and check the homogeneity of the illumination (need to hear the “click” when homing camera). Switch on the scanning mode (FIG A) and take a snapshot (FIG B). The illumination should

homogeneously fill the field of view. If not, re-adjust the MZP and, possibly, the KZP. The scanning image should look similar to the following screenshot:

Fig A

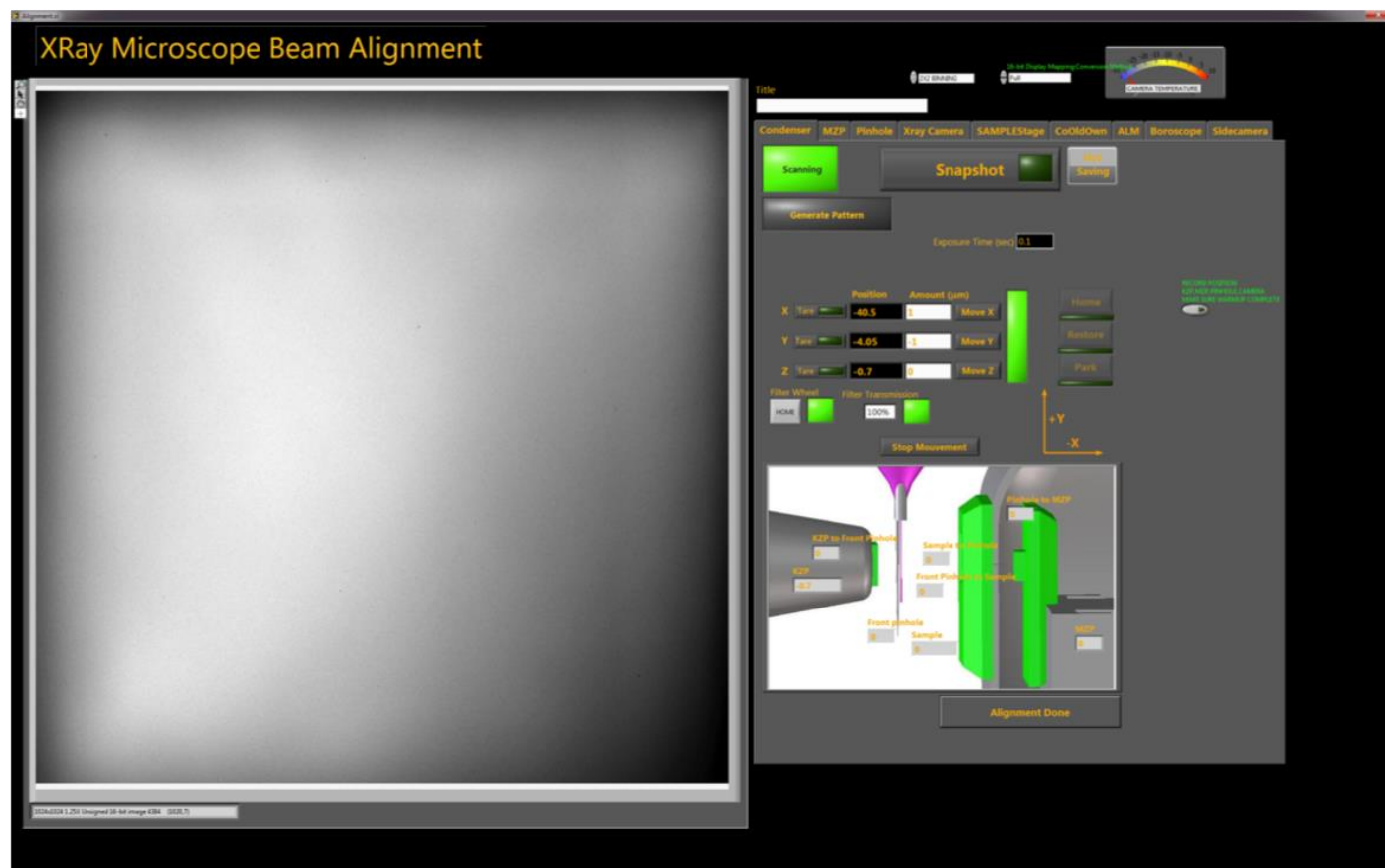
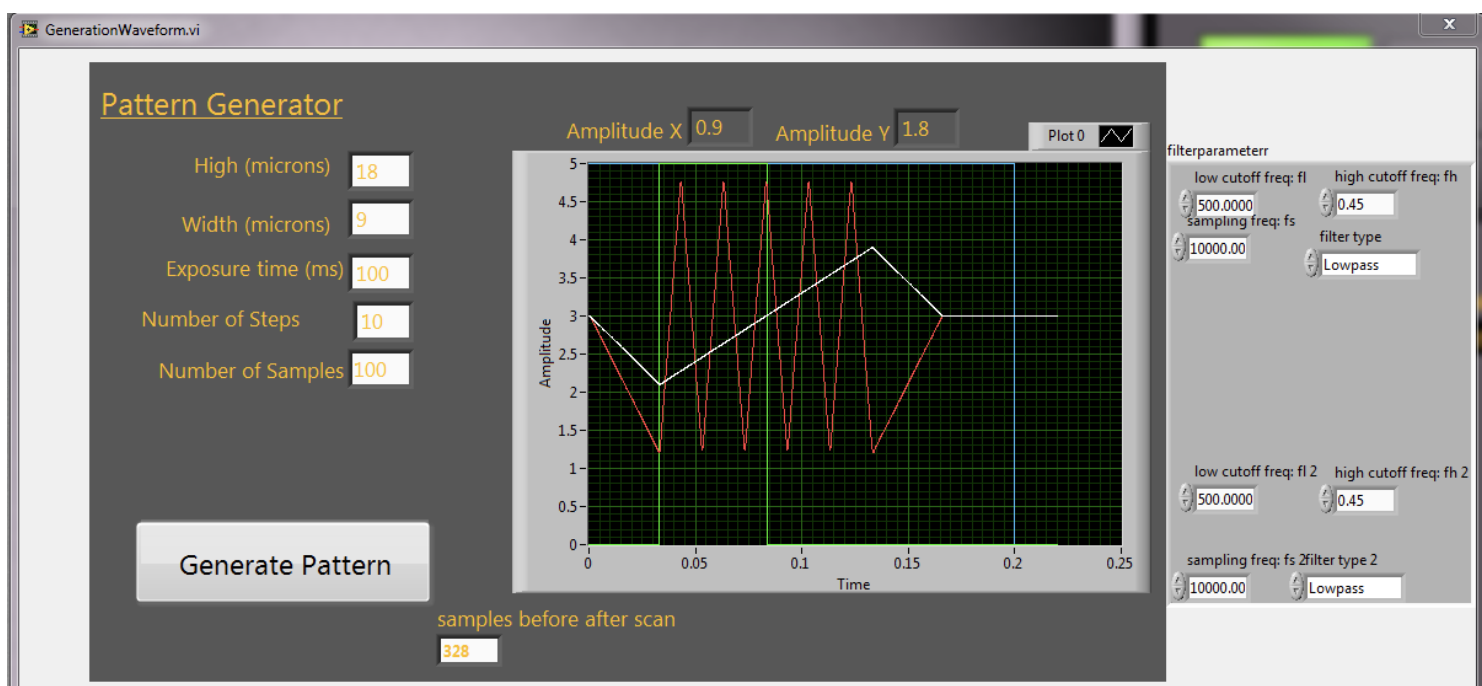


Fig B



7) Press "Alignment done", which closes the window, and "Return to Main Menu". Now proceed with "Specimen selection and loading".

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## Specimen loading and alignment

The pattern generator allows a simple lines scanned pattern. It is defined by the amplitude in X and in Y, the exposure time (time to make the scan), the number of steps and samples. These two last parameters are rarely modified. The graph presents the voltage variations of the different channels that draw the pattern (X in white, Y in red). In green is the pulse that will trigger both camera and the moves of the condenser according to the defined pattern. Here the result with a 100 ms exposure time and pattern of 18 by 18 microns with He flowing

The sample stage has seven motors (regular motion axis X, Y, Z, two inclinations planes XY and XZ and the rotation around the Y axis).

1. To start the acquisition with the right angle
2. Be at the right position for the cryotransfer of the sample.

When the indicator is green, you can press the "Loading New Sample" button.

The Helium flow rises to 2.5 (one the indicator. Voltage is about 5 V), a little column of cold helium can be observed coming out of the sample chamber. The stage moved away automatically by -50000 microns in X to allow room for the cryo transfer machine.

1. Before loading the capillary, you have to grab capillary from the storage box and to replace the transfer device, be sure it makes contact with the hard stops (metal pieces) and screw the side clamp to be sure of the stability of the transfer machine.
2. The clamps that allow the transfer between the machine and the sample stage are opened.
3. The program dialogue asks you to transfer the sample using the transfer machine. When the tube is down, click "Done". Use a bolt to prevent the tube to going too far down. It could contact the back pinhole.
4. The clamps close to grab the tube. The program dialogue asks you to remove the transfer machine. Don't click "Ok" before this is completely done.
5. The stage will move back to the specimen center and go down to approach the tube

The camera of the boroscope is automatically connected to allow you to tune the stage in order to grab the tube. You can watch on the side if the sample holder (two poles) is slightly to the right of the tube. If it is not the case, you need to adjust z with 100 micron step. You need to adjust y by several steps of -1000 microns (between 5 to 7 steps). If you push the tube down, it is certainly because the stage is not at the right place in z. You need to go up a little bit (500 microns are enough) and adjust the stage position by 100 microns step on the z axis.

6. When the stage is grabbing the tube by seral thousand microns, you can click the button "Tube In Position" and clamp the tube with the little spring. Then press Ok. This causes the clamp to release the tube.
7. The GUI automatically switches to the side light microscope allowing a look at the tip and being able to answer these simple questions : Is the tip is ok? (i.e. not too thick, not too icy, not broken). If the tip is broken, click "*Tip Broken*" to take the tube out. You can start the procedure of loading again with a new specimen. To find the tip, you need to move the stage by around +17000 microns in Y.

## **Eucentric alignment procedure (using Rotary Optical Encoder)**

1. Placing the tube in the middle of the green circle screen and in focus once you found
2. Aligning the tip by clicking "ALIGN"
3. The stage will rotate between 0 and 180, click in the middle of the tip, the rotation stage will correct to the center between 0 and 180 degrees, repeat the alignment between 90 and 270
4. Repeat the procedure until it converged to the rotation center
5. When the tube is in focus for every position and stays at the same position. You have well aligned the tube.
8. Before clicking alignment done, place the tube in the middle of the screen with the tip in focus. This will make it easy to find the tube under the X ray Beam.
9. By clicking alignment done, you close the loading module and the stage is going down by -21500 microns. The tube will be close to the position to be imaged with X rays.

## **Deicing**

1. After sending capillary down for x-ray imaging, you have to deice to get rid of the ice around the capillary
2. Click "Tomographic collection" then " Acquire data from current specimen" then "deice"
3. Deicing the sample until -90 degree for 60 secs for the first round, and find the capillary under x-ray
4. Move the capillary down 150um per step for 1200um in total
5. Move the capillary in X axis (left and right) to find the capillary and then align the capillary under x-ray until the rotation converged to the rotation center
6. Once you finished the deicing and found the rotation center, you can start to find a region of interest for x-ray tomography

## **X-ray image alignment**

1. Once you found the cell you want to image, try to make sure that the rotation center is still within the field of view, if not, align the capillary again until it converged.
2. After that, you could start the data acquisition
3. Before taking projections for tomogram, make sure that is binning 2x2, 2 degrees per step for 92 Images, and choose the proper filter for not to saturate the FF images.

You can save snapshot images by toggling the button "not saving". It will change for saving. The images will be saved at C:\essaiimage\Name\_Sample\_Position\_01\_xx.tif. The exposure time box enables a choice between different scanning patterns: simple (exposure time defined by the pattern generator) or double (double exposure time). In this module, you have access to all the motors of the sample stage, X Y, Z axis and the rotation motor. The bottom panel presents the parameters for the acquisition. Number of steps (number of images), the step value (degree). A Pause button available during the acquisition will give to the user the possibility to pause the acquisition and replace the tube in case of misalignment or

drifting of the tube. The button “Beam alignment” opens another window to allow fine tuning of the image. You only have access to the X and Y axis of the KZP and the MZP in this alignment module.

An intensity indicator allows an effective reading of the intensity value of the image disregarding the brightness and contrast chosen by the two blue bars.

A Filter wheel button allows the choice between different neutral filters in order to tune the throughput. (This is only needed for expert in order to check the quality of the optics by comparison with a reference).

## Data acquisition

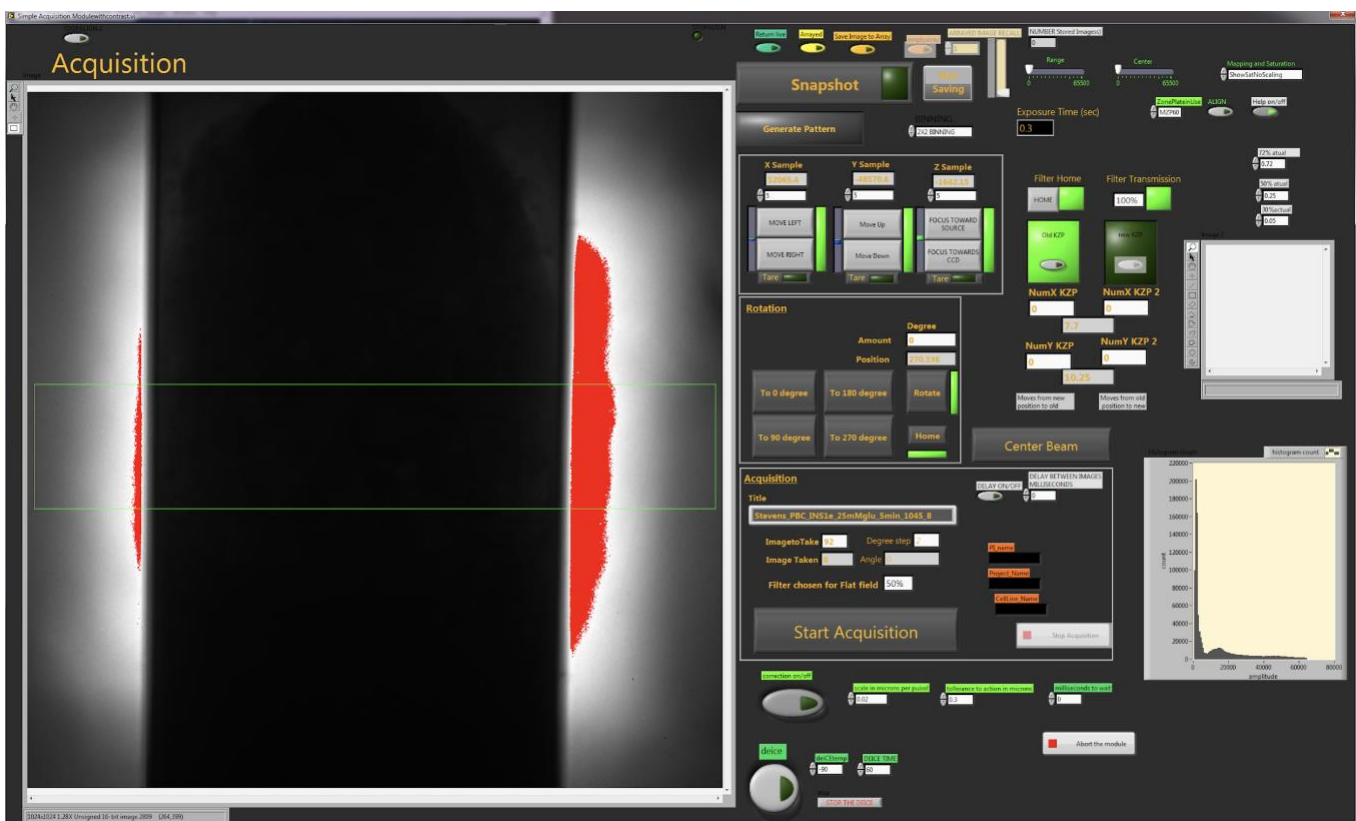
The acquisition module performs several tasks to collect good projection data for tomographic reconstructions. As an example, the projections image data needs to be normalized by a Flat field image (images of the field of view with a single scan with no specimen present). The sequence is as follows:

Before performing the acquisition, set up a region for auto-correction for making sure that the capillary is in the field of view

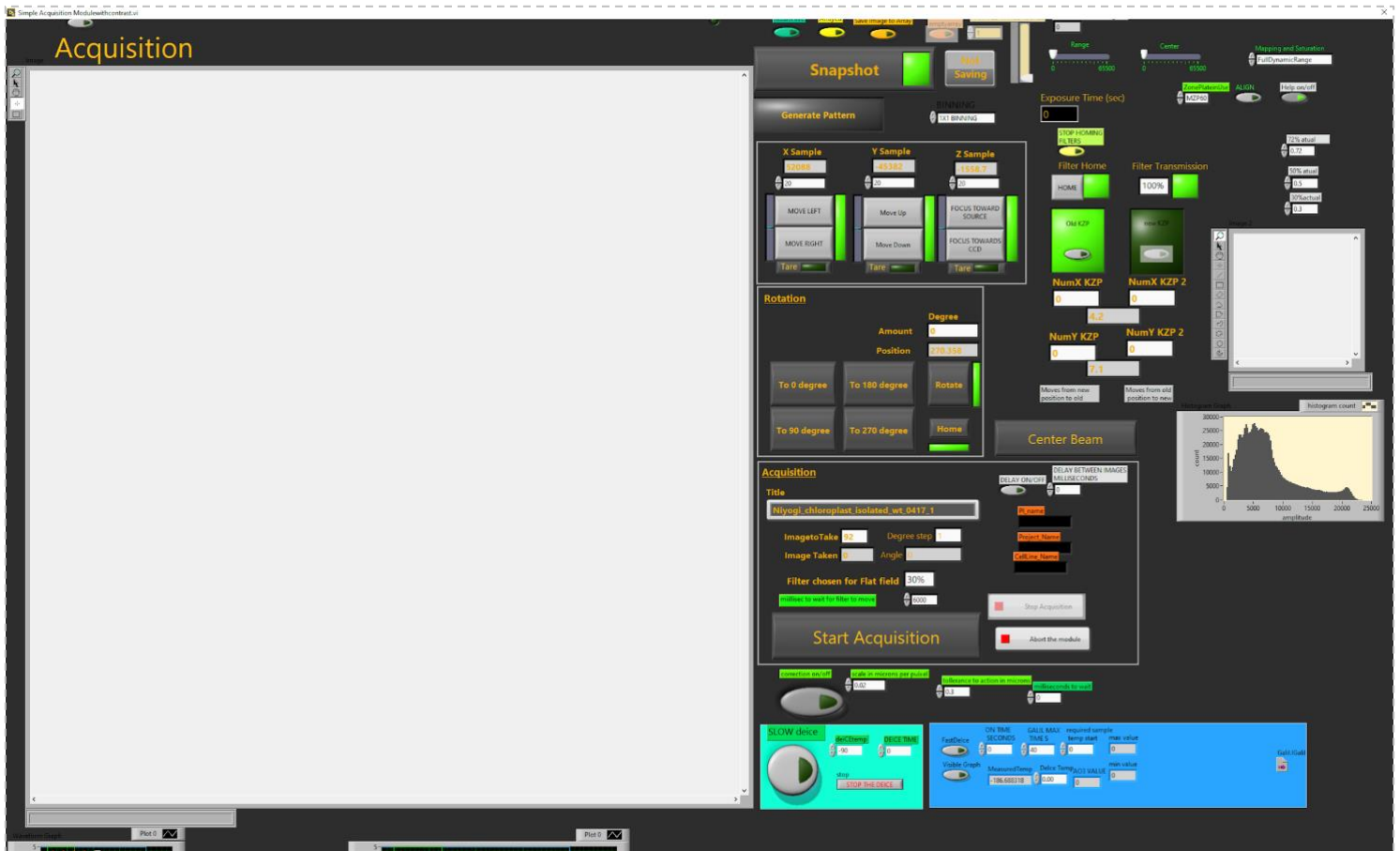
- 1) put a green rectangular in the field of view which includes the capillary
- 2) set up the scale in microns per pixel for 0.017 for 60nm MZP, 0.01 for 35nm MZP
- 3) set up the tolerance range to enable autocorrect (usually 0.3 for 60nm, 0.1 for 35nm)

Make sure all the parameters were set up correct for taking tomogram, the steps for taking projections will be automated once you click “start acquisition”, after starting acquisition,:

- 1) The stage moves away by 40 microns.
- 2) 10 flat field images are taken
- 3) The stage moves back by 40 microns
- 4) The acquisition of your specimen projection data then begins.
- 6) A pop-up window tells you when the acquisition is done. By clicking OK, it will close the right panel.







## Multiple field of view stitching routine

The stitching acquisition module is very similar to the previous one.

Several parameters are selectable, such as the number of field of view you would like to stitch, the displacement in Y between each field of view.

A beam alignment module will open between each acquisition to give time for the user to check the level of nitrogen in the different tank and of course realign the beam in case of drifting.

## Shutdown procedure

1. Before performing shutdown procedure, clicking “Abort the module” then “return to main menu” and then “Microscope start up and alignment” and go to “side camera”
2. Lift the imaged capillary up until it hit the top (along the Y axis of sample stage)
3. Take out the capillary and release the grabber
4. Lower the N2 gas purging to 30
5. Warm up the microscopy “40 degrees for 360 seconds” through the “Deice” procedure
6. Once you reached 10-15 mins warmup, you can stop the “GUI”
7. Shut the microscopy off and shut the heater off

8. Leave the heater on for a few hours
9. Stop thermometer and side camera windows
10. Close N2 and He gas
11. Close the shutter and lock the computer.

Automatic procedure:

By clicking the *Shut down* button, you will launch the warming up and shutting down process.

It will automatically save the position of the pinhole, the MZP and the KZP, this module then parks the KZP and warms up the camera. The module will unload the sample if you forgot to do it after the last acquisition. The Vi will automatically stop when the camera reaches ambient temperature. This is the end of the automatic procedure; manual procedures are required for the remainder of the shutdown.

Manual procedure:

A few things need to be done manually. Decrease the flow the Nitrogen to 0. Be sure that He is flowing in order to flush the microscope with dry helium. You can unplug the cooler controller of the camera when the Shutdown vi stops. You need to wait until the temperature of channel 1 is positive then you can close the He gas cylinder and the Nitrogen bottle. You can shut down the different controllers, power switches, the shutter box and the heater controller. Closing the valve of the zeolite cold trap.

## Overview and Summary

# TROUBLESHOOTING

During data collection you may encounter issues with the microscope. Most can be fixed easily. However, if you are not confident in what you are doing, or have any doubts...STOP and call for help! Whilst precautions have been put in place to prevent injury to you, or damage to the instrument it is impossible to envisage and account for all possible scenarios that can occur in a complex scientific environment. Help is free. On the other hand repairing damaged optics and instruments is expensive. Please don't take chances that endanger your well-being, the safety of others, or the integrity of the instrument.

### ***What if you don't see any specimen illumination light?***

If you don't feel confident contact a staff member. If you are familiar with the microscope and the control system, and confident of your abilities then work through the following list of possibilities:

- Check the EPS windows. Are the shutters opened?
- Check beam is available and the synchrotron ring is operating normally

- Check if the shutter control box is switched on (this is the black box on the left on the optical table)
- Check if the KZP has been restored to the previous position. To do this check the database from where the data are collected and saved (when the microscope is turned off). The database can be found in C:\Database\DatabaseGalil.accdb. It will be opened in Microsoft Access 2010. The table "Galilparameters" can be opened by a double click. You will find the different position of the optical elements according to the date. The last line is the last known position when the microscope was operated. You can directly check if these numbers are consistent with the read positions at the different tabs of the alignment module. If you notice a difference, you can try to move the elements (KZP, Pinhole and MZP) to this previous position. Warning: Don't ever move the MZP along the Z axis. Call Bertrand Cinquin or Mark Le Gros to assist with this problem.
- Go the camera Tab, home it and move it by several steps of -100000 microns (-700000 maximum. It will move the camera toward the head of the line to have a larger view of the optics (you will see some photons of the 0 order passing around the condenser). Call Jian-Hua Chen or Mark Le Gros before proceeding with data collection.

***Some popup windows appeared with the error message ERR-2147353267***

Check the controllers: Maybe some of them are not responding. You need to turn them off and back on again before trying again.

***Some popup windows with the appeared ERR-2147467259***

The instrument is losing connection with one of the controllers. You should restart all of them.

***The GUI is active but clicking buttons does not activate anything***

Labview Vis can't be simply aborted by being shut using the cross (right corner of a windows window). You have to close the different modules using the Done button.