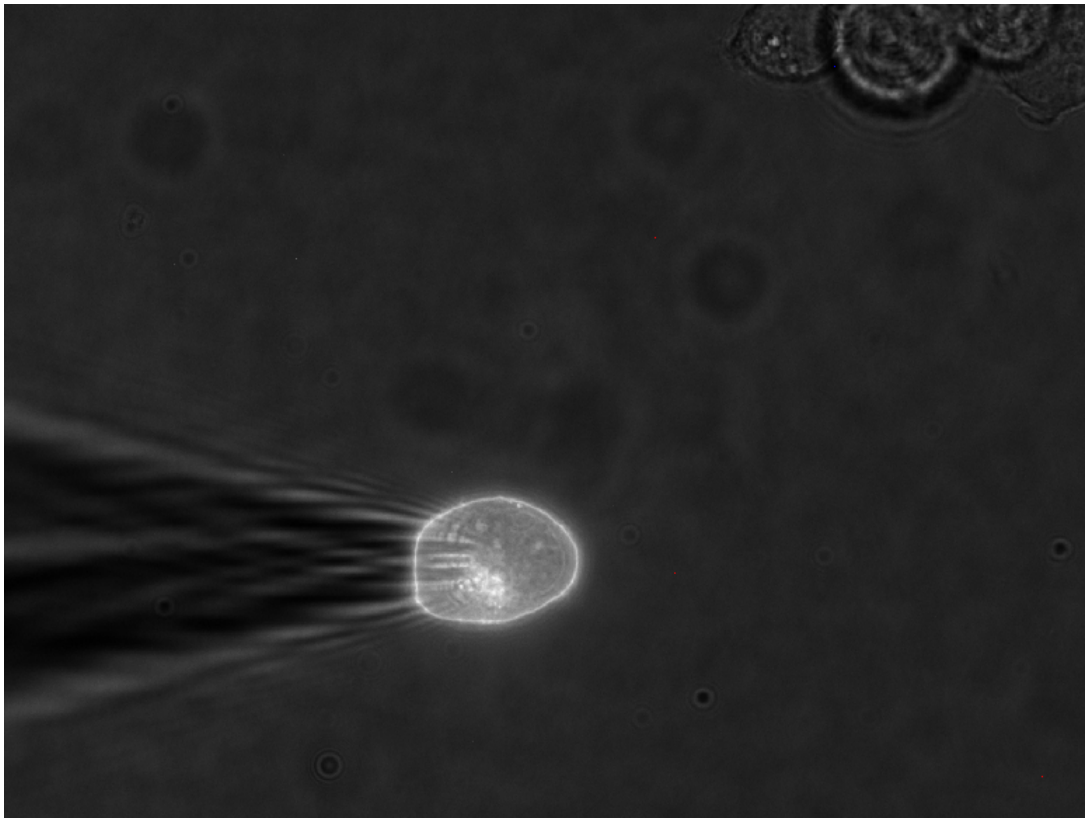
SmartLUX

The Imaging Extension for Electrophysiology & Electrochemistry



User Manual

Version 1.1a

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Title Page: Cell contacted by a patch pipette; Courtesy of Prof. Stefan H. Heinemann, Institute for Biochemistry and Biophysics, Friedrich-Schiller-University, Jena, Germany

COSXMA/3

Contents

1. Introduction

Performing a fluorescence imaging experiment is a quiet complex process. The whole setup needs to be organized and tuned for the experiment which very often requires the integration of numerous hardware and software components. In addition, one should yield experimental recording conditions allowing reproducibility between individual experiments without changing major parameters.

Once you combine fluorescence imaging experiments with electrophysiological recordings, e.g. patch clamp recordings, you would prefer to have both kind of data acquired at the same time, precisely aligned without having numerous and variable delays.

SmartLUX provides an enourmous help to reach this target and to facilitate the combined recording of the patch clamp experiment and the fluorescence imaging. SmartLUX allows to setup and perform simultaneous image acquisition and electrophysiological recordings. As an extension of the versatile Patchmaster software it helps to setup and control the fluorescence excitation light source, the camera and the electrophysiologcial stimuli. These signal get integrated into the Pulse Generator of Patchmaster by simple mouse clicks, thereby taking over responsibility of proper channel definitions and segment timings. Here, Patchmaster, in conjunction with SmartLUX, is the control unit to integrate all necessary hardware settings. This guarantees that all outgoing and incoming signals are synchronized and aligned.

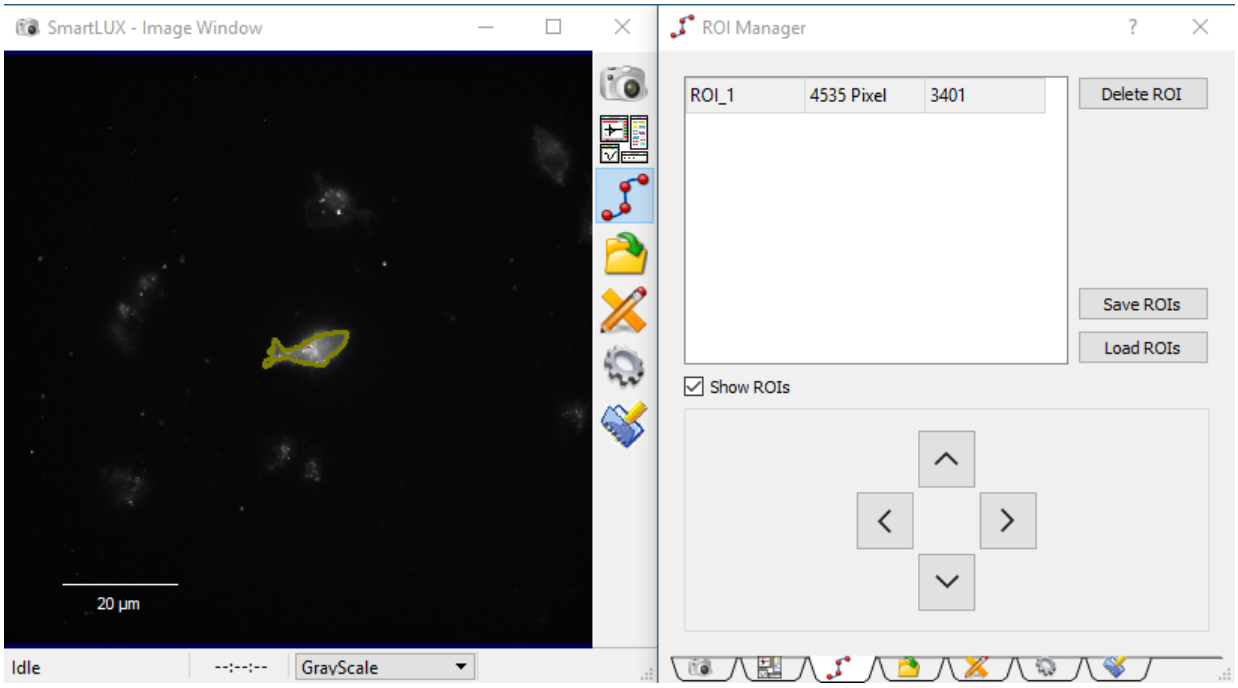


Figure 1.1: SmartLUX, the imaging extension software of Patchmaster.

Fluorescence mean values from regions of interest (ROI), which can be marked by the user in the acquired image, are plotted together with e.g. the current or voltage Traces in the Oscilloscope Window and stored together with the other data in Patchmaster's data tree. When stepping through the data tree, SmartLUX shows the corresponding fluorescence images which means both kind of data sets are still linked with each other.

1.1 Disclaimer

This *Product* relies on the tools of Microsoft Windows 10 (64-bit). HEKA is not responsible for:

1. the contents of these third party products,
2. any links contained in these third party products,
3. changes or upgrades to these third party products and
4. for any consequential damages resulting from the use of these products.

This *Product* may not be reverse engineered, decompiled or disassembled without the express written consent of HEKA.

In no event shall HEKA be responsible for any incidental, punitive, indirect, or consequential damages whatsoever, (including but not limited to loss of data, privacy of data or other pecuniary loss), arising from or relating to the use, or the inability to use, this *Product* or the provision, or lack of provision, of support services.

In all cases where HEKA is liable, the extend of HEKA's liability shall be limited to the actual cost of the *Product* or to the provision of a replacement version of the *Product*.

1.2 Supported Hardware

SmartLUX does currently support cameras of the following manufacturers:

- Andor
  - iXon
  - Neo
  - Zyla
- Hamamatsu
  - Orca Flash 4.0
  - Spark
- PCO
  - PixelFly
- Photometrics
  - Prime 95B
  - Evolve 512 Delta
  - CoolSnap
  - BSI
  - BSI Express
- QImaging
  - OptiMOS
  - Retiga Electro

Note: *We continuously expand the supported camera models. Please contact us to find out if your camera is already supported or will be supported in future release versions.*

1.3 Supported System Software

SmartLUX is supported on MS Windows 10 (32-bit or 64-bit).

2. Installation

Before installing the SmartLUX software you need to install the Patchmaster software first. Further, the dongle driver for the USB HASP dongles need to be installed. Detailed instructions to install Patchmaster and the dongle driver can be found in the "Installation Guide for HEKA Hardware and Software" which can be downloaded here.

Together with your SmartLUX dongle you should have received the SIDX license (printed on a piece of paper). If you are missing any parts please contact your local sales representative.

The latest software version of SmartLUX can always be downloaded from the HEKA Website: SmartLUX Download.

Download the zipped folder and extract the content. The extracted folder contains the following files:

- lut folder: Contains all look up tables for pseudo-colors
- ReadMe.text file: A text file which contains installation instructions
- SIDX.dll file: Dynamic program library
- SmartLUX.exe file: SmartLUX software

Open the `ReadMe.txt` file and follow the instructions. For clarity we repeat the individual steps written in the text file.

1. Copy the files `SmartLUX.exe` and `SIDX.dll` into your local folder which is by default located in `C:\Program Files (x86)\HEKA\Patchmaster\`.
2. Please create a file called `license.txt` in the folder `C:\Program Files\Bruxton\SIDX\`. If the folder does not exist please create it. Open the text file and copy the following line into the file:

   ```
   <vendor>Bruxton Corporation</vendor><license>XXXXX-XXXXX-XXXXX-XXXXX-XXXXX</license>
   ```

   Replace the "XXXXX" characters with your individual Bruxton license key.

Now, you can start the Patchmaster software.

3. Starting PATCHMASTER

After starting PATCHMASTER, open the Configuration window (Hardware tab) and activate SMARTLUX by selecting the appropriate light source for the imaging experiment. The `Imaging Extension` has to be selected to support the fluorescence excitation light source of your setup.

SMARTLUX supports the following fluorescence excitation light sources (or light source which be controlled in the same way):

- TILL Photonics (Polychrome and Oligochrome) / Cairn (Optoscan)
- DG-4/DG-5 wavelength switcher from Sutter Instrument Company
- Lambda-10 filter wheels from Sutter Instrument Company
- DeltaRAM monochromator from pti
- no device: Use this to handle the excitation light source manually

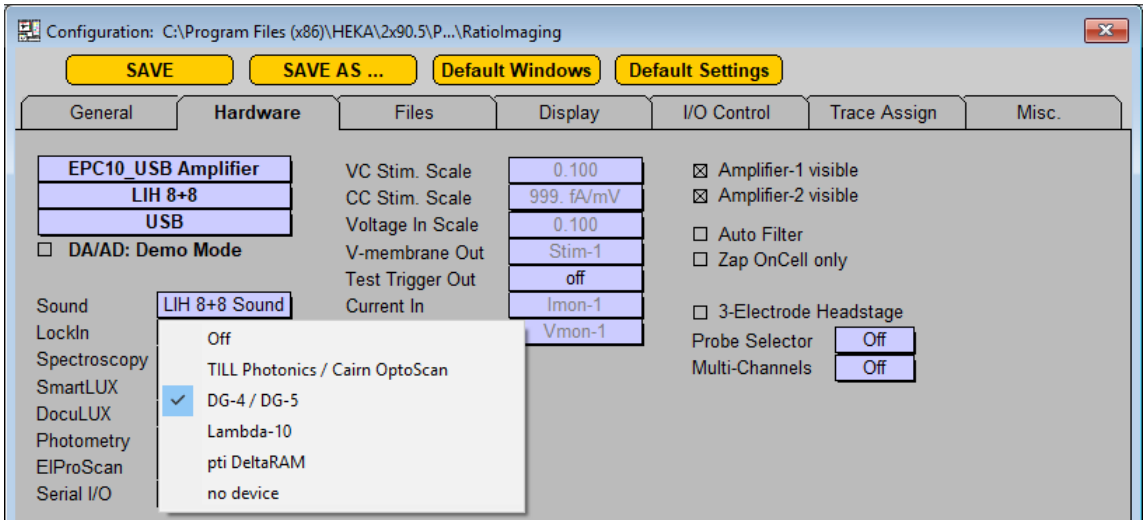


Figure 3.1: Activating SMARTLUX in the Configuration of PATCHMASTER.

After activating SMARTLUX, the Imaging settings dialog of PATCHMASTER for the corresponding excitation light source is opened. Further, the Image Control window and the Image Window of SMARTLUX pop up.

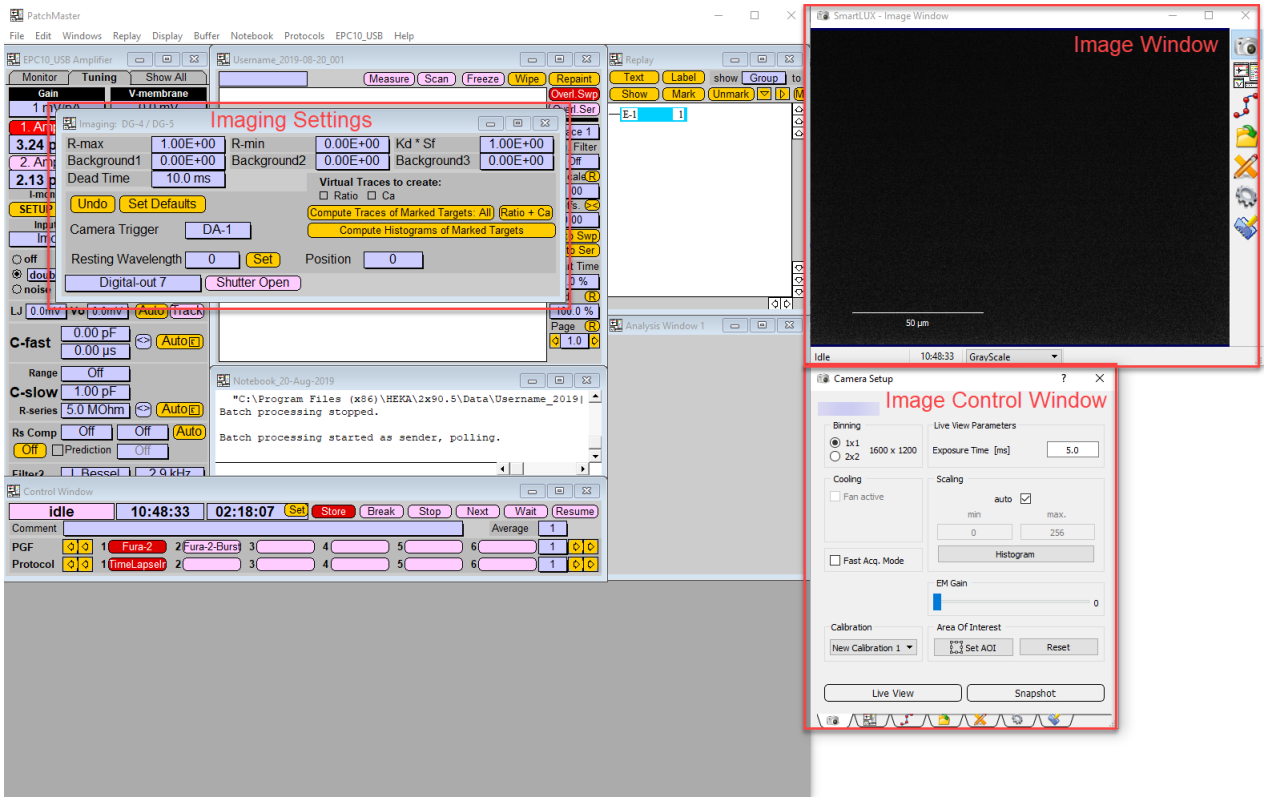


Figure 3.2: Upon activation of SmartLUX the Imaging settings dialog, the Image Window and the Image Control Window open.

In the following chapters we will describe the different settings and options for all three windows.

4. Light Source Configuration

This section provides an overview of the settings for each of the fluorescence excitation light source used with SmartLUX.

Once the SmartLUX has been activated the Imaging window can be opened from the `Windows` menu of Patchmaster.

4.1 General Imaging Parameters (No Device)

Some controls are common for all configurations describing the different fluorescence excitation light sources. These controls are described in this section and the list of parameters equal the selection "No Device" in the Configuration window of Patchmaster.

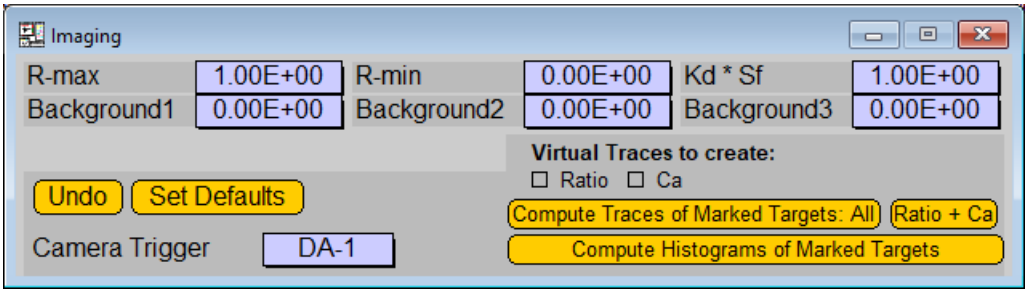


Figure 4.1: Available Imaging Parameters with "No Device" selected.

R-max: Maximum ratio value (F1/F2) in your experimental setup. To get this value you need to measure the minimum ratio under Ca^{2+} free recording conditions.

R-min: Minimum ratio value (F1/F2) in your experimental setup. To get this value you need to measure the maximum ration under saturating Ca^{2+} conditions.

Kd*Sf: Enter the effective Kd (dissociation constant) of the fluorescence dye.

> ***Note:*** *These values are required for calculating the free Calcium concentration (according to the Grynkiewicz formalism). The Calcium concentration can be stored as a Trace by use of the "Photo_Ca" input channel in the Pulse Generator dialog. You need to calibrate your system to get each individual value.*

Background 1...3: Background fluorescence for the three possible excitation wavelengths: F1, F2, F3. The background values are stored as Series parameters with the raw data file and are listed in the Parameters dialog of Patchmaster.

Camera Trigger: Select the output channel which is used for triggering the camera.

Virtual Traces to create: Check the Traces (either for the Ratio or the Calcium Concentration) which should be automatically generated by the Pulse Generator for data acquisition or created by the *Recompute* function (see below).

Compute Traces of Marked Targets: ALL: Recomputes the complete fluorescence data set starting with the calculation of the mean fluorescence values from the selected regions of interest (ROIs). Use this function when you have modified the set of ROIs.

Compute Traces of Marked Targets: Ratio + Ca: Recomputes the *Ratio* and *Ca* Traces which are based on the fluorescence values stored in Patchmaster. Use this function when you modify the *Background* values or the calibration *Parameters* for the calculation of the calcium concentration *R-min*, *R-max*, *Kd*Sf*.

Compute Histogram of Marked Targets: A histogram Trace is created for every image connected with your recordings and added at the end of the Replay tree. If you have drawn a ROI in your image the histogram is created only for the ROI and not the total image. When several ROIs exist PATCHMASTER will create histogram Traces for every ROI.

4.2 TILL Photonics / Cairn

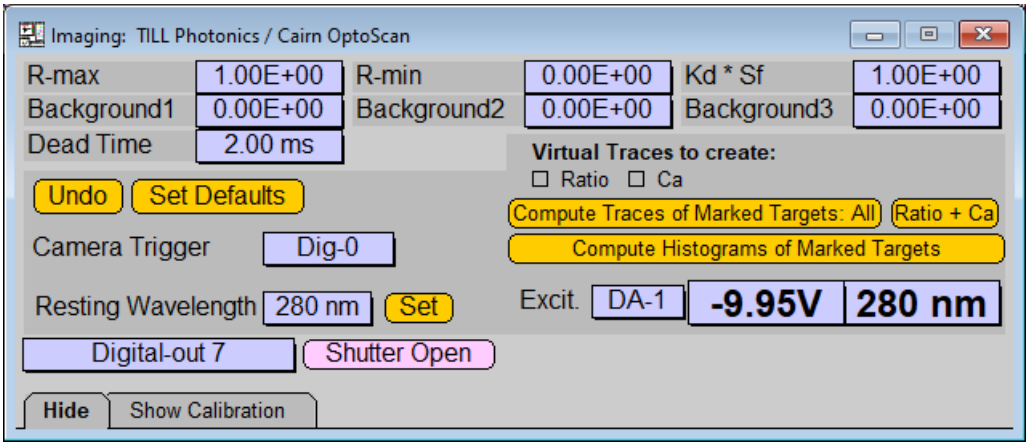


Figure 4.2: Available Imaging Parameters with *TILL Photonics/Cairn* selected.

Dead Time: Time to allow for settling of the commanded wavelength before starting the image exposure.

Resting Wavelength: Wavelength to be set during resting periods.

Set: Only after pressing the *Set* button the wavelength entered in the *Resting Wavelength* field is output.

Excit.: Select the output channel for the voltage output (free analog or digital output). This function is used to control the monochromator. Once it is calibrated, the output can be defined either in [V] (left) or in [nm] (right).

Shutter Selection:

- No Shutter: No shutter available.
- Shutter Control: Shutter can be closed/opened via a button.
- Digital-out 7: Shutter control via digital output.

4.2.1 Wavelength Calibration

Important note: *The following calibration options are applicable for TILL Polychrome devices only.*

In this area the monochromator is calibrated, i.e. the relationship between output voltage and the wavelength is defined. To do so, voltage ramps ranging between the values specified by *from... to* are output when the *Scan* buttons are pressed. This is done for two bandpass filters with known transmission maxima. The corresponding wavelengths are entered in the *wavelength* fields. After both scan operations, i.e. when the voltages corresponding to the peak transmission have been determined successfully, the calibration constants for the control of the monochromator are calculated upon *Compute Factors* is pressed.

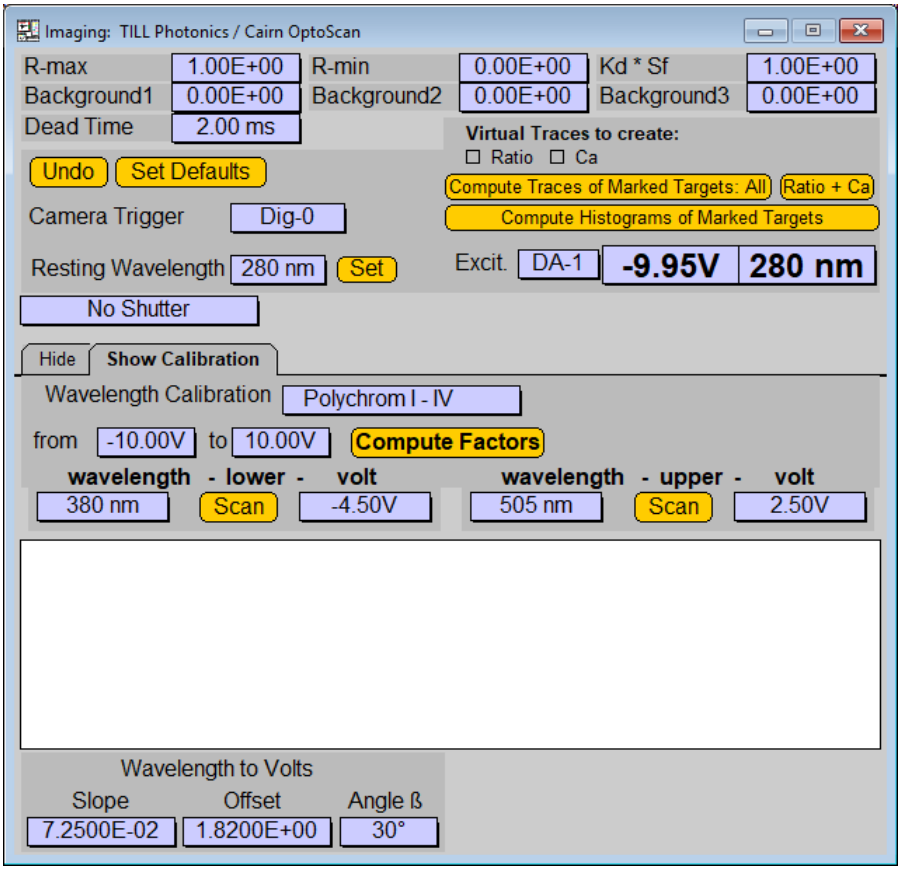


Figure 4.3: Calibration Options of the *TILL Photonics/Cairn* Excitation Light Source Selection.

- Wavelength Calibration: The various *Polychrome* devices are separated into two different calibration groups:
  - Polychrom I-IV
  - Polychrom V
- from / to: Scan range for voltage ramps. For a single bandpass filter usually the full range of ±10 V can be scanned. However, when a single multi-band filter is used, the scan range has to be limited in order to detect only the peak of interest.
- Compute Factors: Compute calibration constants based on the determined relationships of wavelength and voltage.
- lower wavelength: Enter here the maximum of the bandpass filter [nm].
- lower Scan: Do the scan for the low wavelength calibration filter.
- lower Volt: This field shows the voltage corresponding to the peak transmission.
- upper wavelength: Enter here the maximum of the bandpass filter [nm].
- upper Scan: Do the scan for the high wavelength calibration filter.
- upper Volt: This field shows the voltage corresponding to the peak transmission.

4.2.2 Calibrating different TILL monochrometers

Polychrome I and Polychrome II: Two bandpass filters are provided with the Polychrome I and II. Enter the peak wavelength in the *Wavelength* fields of the *Wavelength Calibration* section, then put the first filter in place and press the left *Scan* button. The voltage of the peak intensity will be automatically detected and shown in the

Volt field. Now, put the second filter in place and press the right *Scan* button. Finally, press *Compute Factors* to calculate all calibration parameters.

Polychrome IV: The Polychrome IV comes with a triple-band filter. When using this filter the scan range to detect the first or the last peak of the three has to be limited. Therefore, you should limit the scan range for the first peak from -10 V to -2 V and for the third peak from +2 V to +10 V. We omit the center peak in this calibration. Please enter the peak wavelengths in the corresponding *Wavelength* fields and the scan range before performing each scan in the *from - to* fields. Then, perform the two scans and finally, press *Compute Factors* to calculate all calibration parameters.

Polychrome V: The Polychrome V features an auto-calibration. Please ask TILL Photonics for a pair of calibration values. E.g. which analog control voltages refer to the wavelength 380 nm and 505 nm. Then, please enter the wavelength and voltages in the *Wavelength* and *Volts* fields and press *Compute Factors* to calculate all calibration parameters.

4.3 DG-4/5

Note: *To control the DG4/DG5 via the* SmartLUX *software you need a special cable connecting the Digital Outputs of the interface and the DG4/DG5. Please contact HEKA (sales@heka.com) if you need such a cable (Order Number: 895102).*

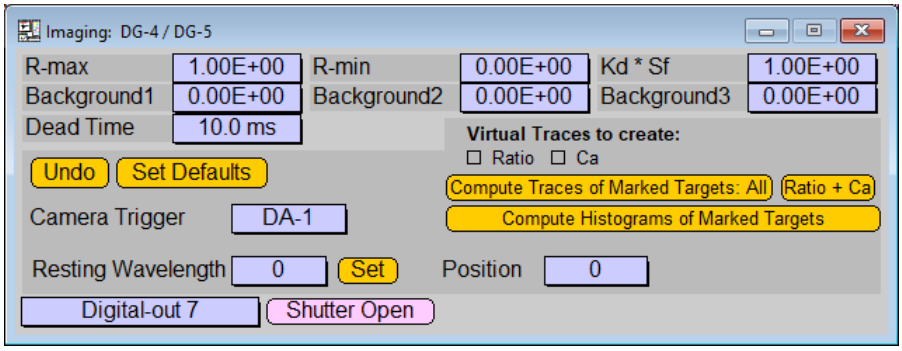


Figure 4.4: Available Imaging Parameters with *DG-4/5* selected.

Dead Time: Time to allow for settling of the commanded wavelength before starting the image exposure.

Resting Wavelength: Wavelength to be set during resting periods.

Set: Only after pressing the *Set* button the wavelength entered in the *Resting Wavelength* field is output.

Shutter Open/Close: Toggles between an open and a closed shutter.

Shutter Selection:

- No Shutter: No shutter available.
- Shutter Control: Shutter can be closed/opened via a button.
- Digital-out 7: Shutter control via digital output.

Position: Enter a number for the *Filter Position* between “0” and “15”.

4.4 Lambda-10

Note: *To control the Lambda-10 via the* SmartLUX *software you need a special cable connecting the Digital Outputs of the interface and the Lambda-10. Please contact HEKA (sales@heka.com) if you need such a cable (Order Number: 895102).*

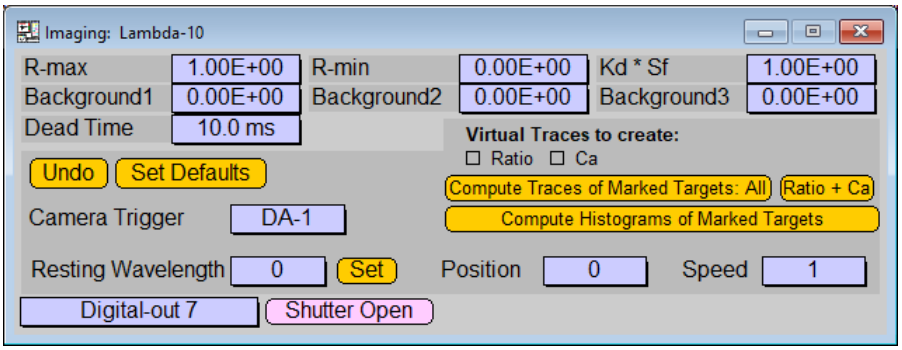


Figure 4.5: Available Imaging Parameters with *Lambda-10* selected.

Dead Time: Time to allow for settling of the commanded wavelength before starting the image exposure.

Resting Wavelength: Wavelength to be set during resting periods.

Set: Only after pressing the *Set* button the wavelength entered in the *Resting Wavelength* field is output.

Shutter Open/Close: Toggles between an open and a closed shutter.

Shutter Selection:

- No Shutter: No shutter available.
- Shutter Control: Shutter can be closed/opened via a button.
- Digital-out 7: Shutter control via digital output.

Position: Enter a number for the *Filter Position* between "0" and "15".

Speed: Set the filter wheel speed of the *Lambda-10* between "0" (fast) and "7" (slow).

4.4.1 Using a Lambda-10-3

Before using the Lambda-10-3 controller for controlling the shutter and the filter wheel via the PatchMaster software you need to set the Lambda-10-3 controller into "Parallel Port" mode. By default the Lambda-10-3 controller is configured for "USB" mode. To set it into the "Parallel Port" mode you need to press the following keys at the controller:

- Switch on the device
- Press "Local"
- Press "Mode"
- Press "7 - Default"
- Press "1 - Set the new default"
- Press "4 - Comm Port"
- Press "3 - Parallel Port"

- Press “0 - Set no other defaults”

Once this prerequirement is fulfilled there are two reasonable ways to control the shutter via the Lambda-10-3:

1. Using the digital output channel “Dig-7” controlled in the Imaging dialog ⇒ TTL Mode
2. Using the digital bit (word) commands either in the Pulse Generator, Protocol Editor or I/O Control Window ⇒ Conditional Mode.

4.4.1.1 TTL Mode

To control the shutter via the “Dig-7” digital port you need to enable “TTL” mode at the Lambda-10-3 controller:

- Switch on the device
- Press “Local”
- Press “Mode”
- Press “3 - TTL”
- Press “Enable TTL”

If the “TTL” mode at the Lambda-10-3 is disabled it is not possible to use SmartLUX to control the shutter.

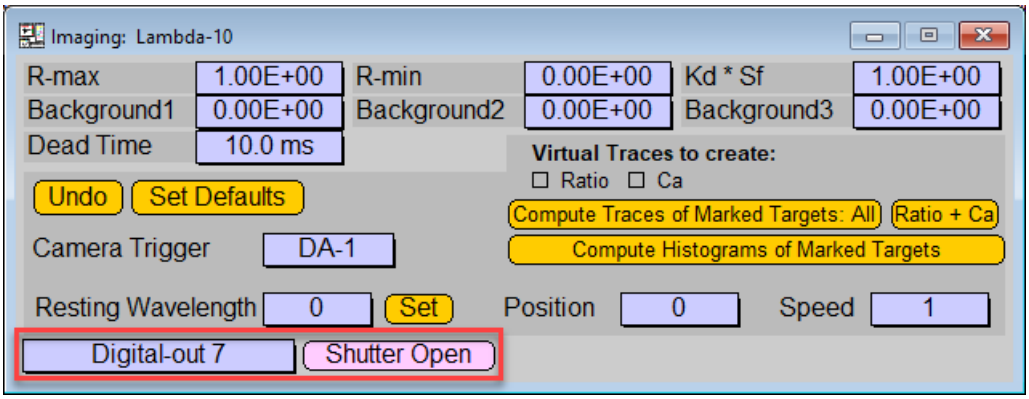


Figure 4.6: Shutter control via the digital output port “Dig-out 7”.

Now, one can use the *Shutter* button in the Photometry Dialog or the digital output channel “Dig-7” in the I/O Control Window or elsewhere to control the shutter.

4.4.1.2 Conditional Mode

To control the shutter in the *Conditional Mode* you need to disable “TTL” mode at the Lambda 10-3 controller:

- Switch on the device
- Press “Local”
- Press “Mode”
- Press “3 - TTL”
- Press “Disable TTL”

One can activate the "Conditional Mode" of the Lambda-10-3 e.g. in the I/O Control Window and enter the bit "299" (deselect *Single Bit* first). This means the shutter closes automatically when the filter wheel is moved and opens again when the filter wheel movement has finished.

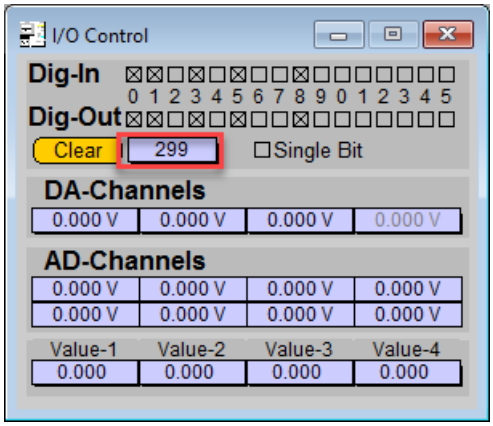


Figure 4.7: Setting digital bits manually via the I/O Control Window of PATCHMASTER.

To switch off the "Conditional Mode" you need to send either bit "298" or bit "300". Bit "298" disables the "Conditional Mode" but leaves the shutter open, bit "300" disables the "Conditional Mode" but closes the shutter.

4.5 pti DeltaRAM

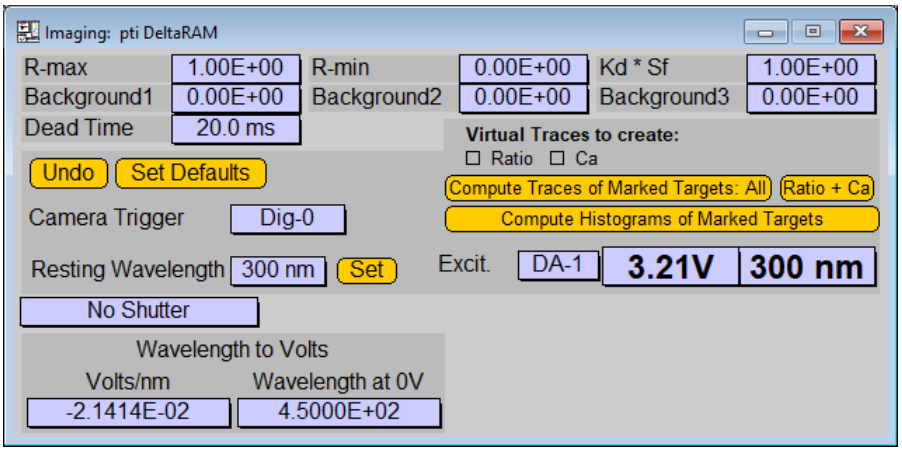


Figure 4.8: Available Imaging Parameters with *pti DeltaRAM* selected.

Resting Wavelength: Wavelength to be set during resting periods.

Set: Only after pressing the *Set* button the wavelength entered in the *Resting Wavelength* field is output.

Excit.: Select the DA channels for the voltage output. This function is used to control the monochromator. Once it is calibrated, the output can be defined either in [V] or in [nm] (right).

Shutter Selection:

- No Shutter: No shutter available.
- Shutter Control: Shutter can be closed/opened via a button.
- Digital-out 7: Shutter control via digital output.

Wavelength to Volt: *Volts/nm* and *Wavelength at 0 V* are internal calibration parameters, which must be given as described in the specifications of your DeltaRAM system. The PTI system has a linear relationship between the applied voltage and the wavelength of the excitation light.

4.6 Digital-Trigger-based Light Sources

In some cases one does have other triggered light-sources like LED light sources or lasers. This chapter provides an overview how to use such a light source together with SmartLUX.

Connect the digital outputs of the EPC 10 USB via BNC cables to your light source.

Use the I/O Control Window to manually test the proper activation and deactivation of your light source by using the digital word command.

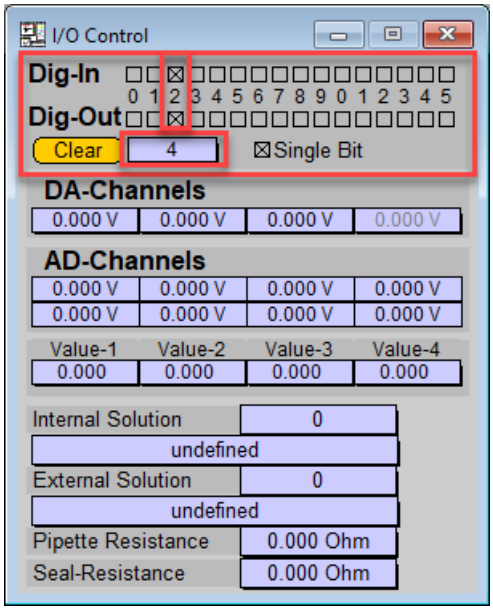


Figure 4.9: I/O Control Window of Patchmaster for testing of the digital-trigger-based light source used in SmartLUX.

The table below lists the first four digital outputs and the corresponding *Digital Word* command to activate the light source.

Table 4.1: *Digital Word* command for SmartLUX to activate a digital-trigger-based light source with four digital output channels.

Dig-Out 0	Dig-Out 1	Dig-Out 2	Dig-Out 3	Digital-Word in SmartLUX or PATCHMASTER
0	0	0	0	0
1	0	0	0	1
0	1	0	0	2
1	1	0	0	3
0	0	1	0	4

Once you made sure that the triggering of the device works fine you activate SmartLUX in the Configuration of Patchmaster by either selecting `DG-4/DG-5` or `Lambda-10` for the light source. The reason is, that both devices

can be digitally controlled. Hence, the provided settings are suitable for our digitally-triggered light source. In the further description we use the DG-4/DG-5 selection.

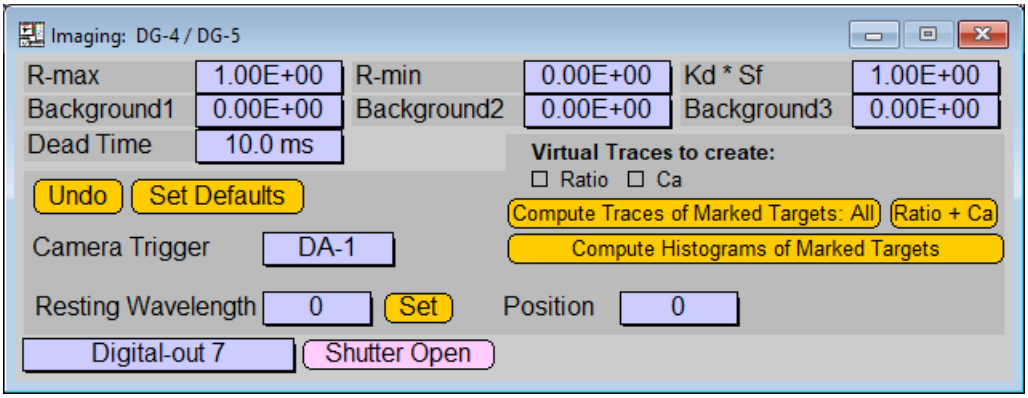


Figure 4.10: DG-4/DG-5 imaging parameters of PATCHMASTER.

Set the *Dead Time* to "1 ms" and select either "DA-0" or "DA-1" for the *Camera Trigger* channel. The digital channels are not available and are used to control the light source.

In the `PGF Primer` dialog of SMARTLUX you can now enter the digital bit number (digital word) instead of the *Wavelength* to address the connected light sources.

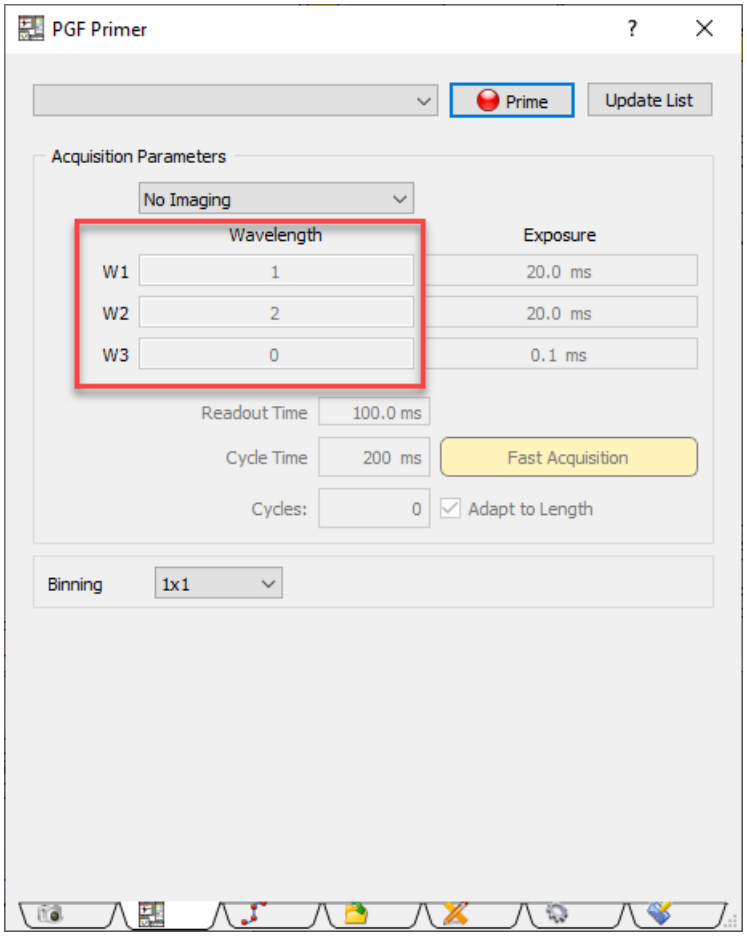


Figure 4.11: `PGF Primer` dialog of SMARTLUX.

5. Image Window

The `Image` window displays the images during image acquisition or when loading an image file.

The icons on the right side of the window are shortcuts to the corresponding tabs of the Image Control window. They are explained in detail in chapter 6 on page 19.

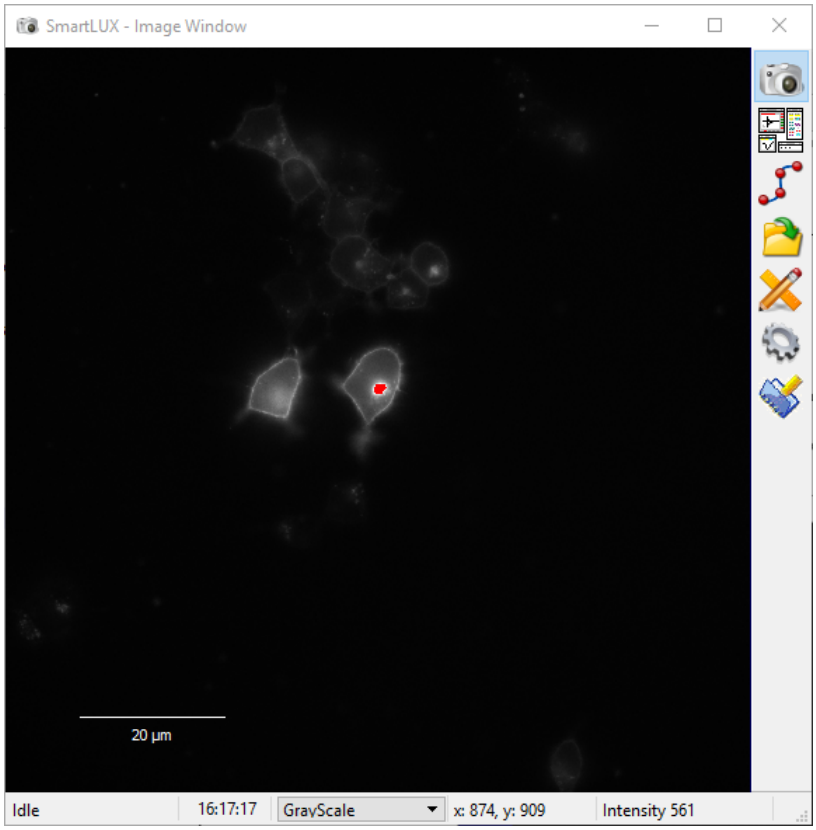


Figure 5.1: Image Window of SmartLUX.

At the bottom of the Image Window you can get some further information (starting from left to right):

- Status
  - Acquiring: x of x: Indicates how many images/frames were acquired and the total number.
  - Idle: Nothing happens
  - Initializing: SmartLUX is initializing
  - Loading File: SmartLUX is loading an image file.
  - Recomputing: Fluorescence intensity values are currently recomputed.
  - Replaying: Images from an image file (TIFF stack) are currently replayed.
- System Time: Displays the time of the operating system. If no time is displayed then there is no synchronization between SmartLUX and Patchmaster.
- Lookup Tables: Selection list for different pseudo-color or grey-scale settings of the displayed image.
- Pixel Position: Shows the pixel position of the mouse tip (X- and Y-value).
- Intensity Value: Shows the pixel intensity

Context Menu: Upon right-clicking on the displayed image in the Image Window the mouse tip changes into a cross-line. While pressing the right mouse button you are now able to measure structures in your image. The scaling depends on the settings made in the calibration settings (see chapter 6.5 on page 27).

6. Image Control Window

The Image Control Window is the central part of SmartLUX. Here, most of the settings for the imaging process are defined and transfered to the PatchMaster software. The Image Control Window is structured into different tabs. In the following sections we explain the purpose of each individual tab and their parameters.

6.1 Camera Setup

The `Camera Setup` tab contains all parameters and settings provided by the camera itself. Further, you can set the used image calibration and the area of interest.

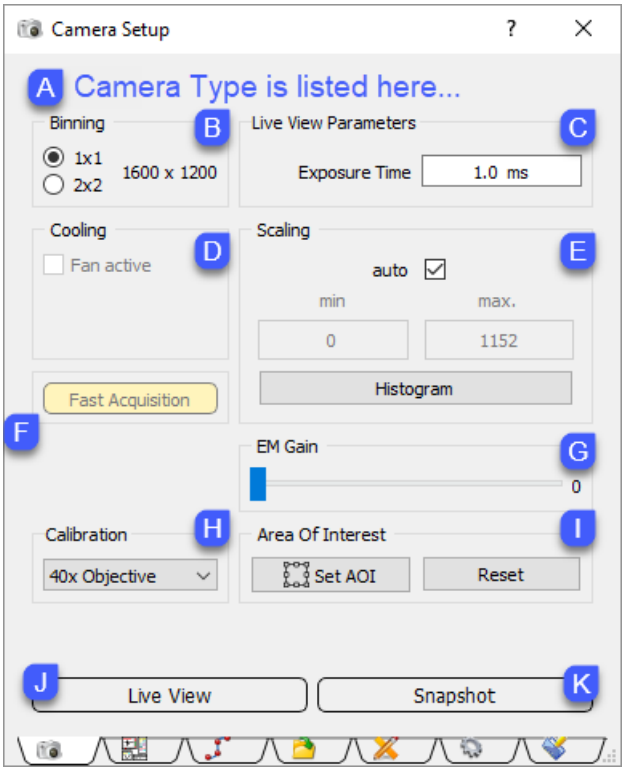


Figure 6.1: `Camera Setup` settings of the Image Control Window.

6.1.1 Camera Reference (A)

Lists the identification of the camera used by SmartLUX. We blured that area to hide the camera identity.

6.1.2 Binning (B)

Allows to set the camera binning factor. The resulting pixel dimensions of the image are shown to the right. The provided values depend on the camera specifications.

> ***Note:*** *The binning factor is typically set once for a complete experiment.*

6.1.3 Live View Parameters (C)

Camera parameters used during *Live View* mode only are depicted here.

Exposure Time [ms]: Set the *Exposure Time* for live image acquisition. Changes can be best monitored when *Live View* is used.

6.1.4 Cooling (D)

If any cooling options are available they are listed here (e.g. “Fan active” or “Water Cooling”).

6.1.5 Scaling (E)

Sets or displays the scaling options of the acquired images or the live image.

auto: When activated each image is scaled with respect to the darkest and brightest pixel.

min / max: When *auto* is activated, *min/max* display the intensity values of the darkest and the brightest pixel. When *auto* is not active, the user can set intensity values for the darkest (black) and brightest (white) pixel manually. For a 16-bit camera the intensity values range from “0” to “65535”.

Histogramm: Calculates an intensity histogram of all pixels and shows it in a separate graph (see below). With a left mouse click on gets a cross line which can be used to scan the histogram for individual intensity values and their corresponding number of pixels.

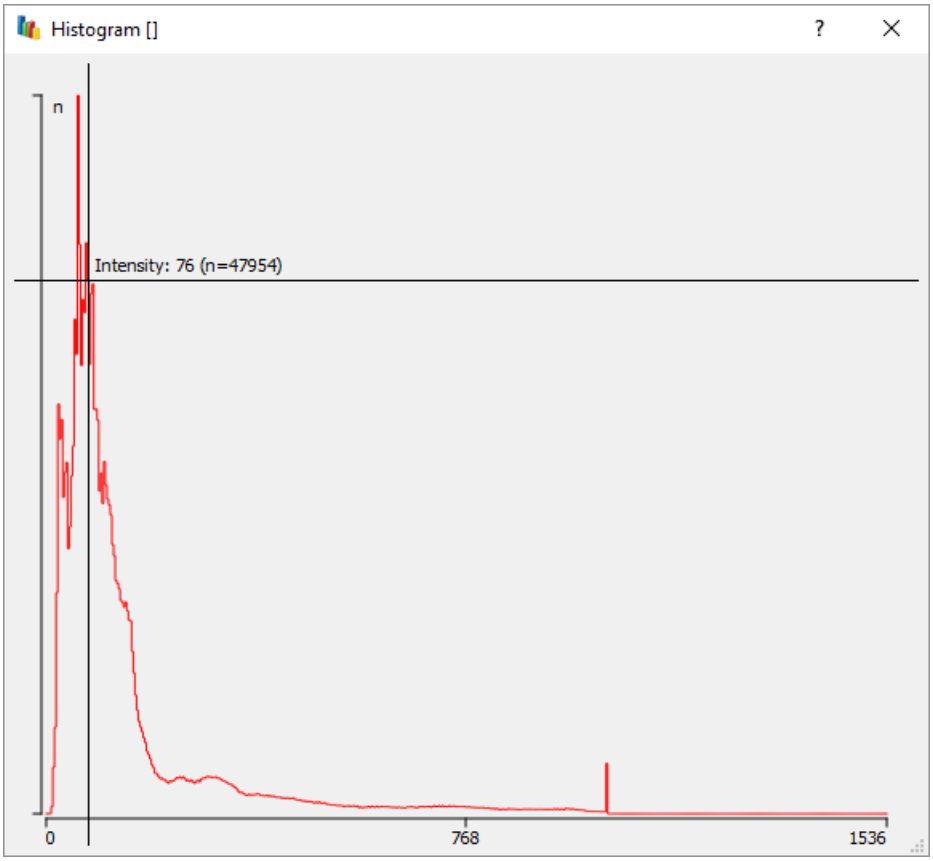


Figure 6.2: Intensity Histogram of an image provided by SmartLUX.

6.1.6 Fast Acquisition Mode (F)

Whenever SmartLUX is operating in *Fast Acquisition* mode it will be indicated here. This is a display only field. *Fast Acquisition* mode is automatically enabled if the acquisition frequency gets faster than 10 Hz. Once activated the user will not see any images or data displayed during the recording to save resources for accurate and fast data acquisition. Further, a recomputation is needed to store the fluorescence data in the Patchmaster Replay Tree.

6.1.7 EM Gain (G)

If available, you can set/adjust the electron multiplying factor of the camera here. It is an amplification of the signal before it gets digitalized. This helps especially under low light recording conditions and improves there the noise-signal ratio.

6.1.8 Calibration (H)

SmartLUX allows to configure and save different calibration settings which might be useful e.g. when using different objectives or cameras. Select the calibration set of your choice from the selection list. Further information about the image calibration can be found in chapter 6.5 on page 27.

6.1.9 Area of Interest (I)

Limiting the image section to areas of your interest is extremely helpful for the experimental procedure. You can i.e. either exclude cumbersome via narrow

Set AOI: By clicking on the button *Set AOI* another dialog called Set Area of Interest opens. This dialog provides the following options:

- X: X-position of the area of interest in respect to the full view image
- Y: Y-position of the area of interest in respect to the full view image
- Width: Pixel value of the width of the area of interest
- Height: Pixel value of the height of the area of interest
- Reset: Resets the area of interest to the default settings
- Cancel: Aborts the selection of the area of interest
- OK: Confirms the selected area of interest

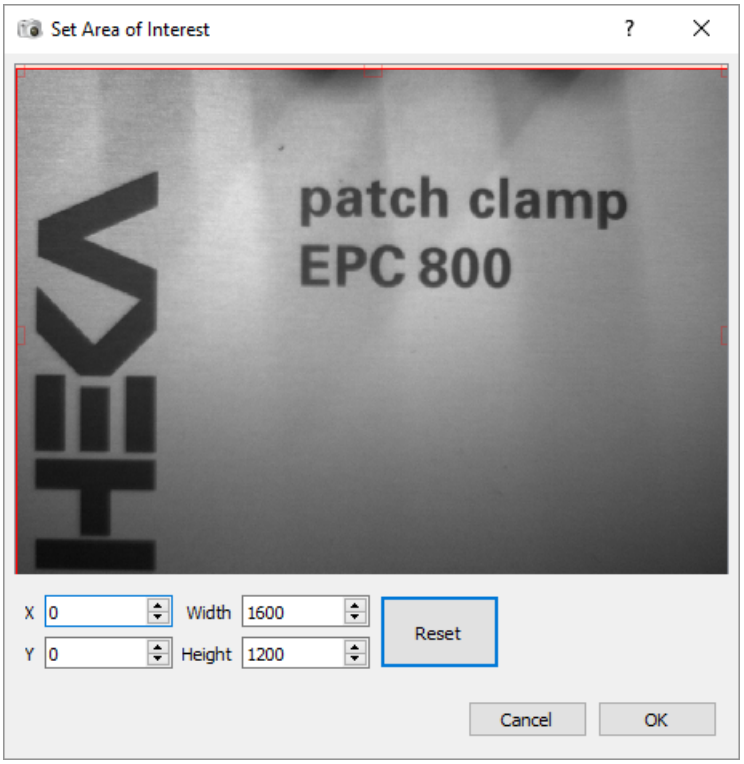


Figure 6.3: Set Area of Interest dialog with full view image.

The picture above shows a full view snapshot of your specimen. While narrowing (use the red cursors lines for image size definition) the area for the imaging process you can increase the imaging speed considerably.

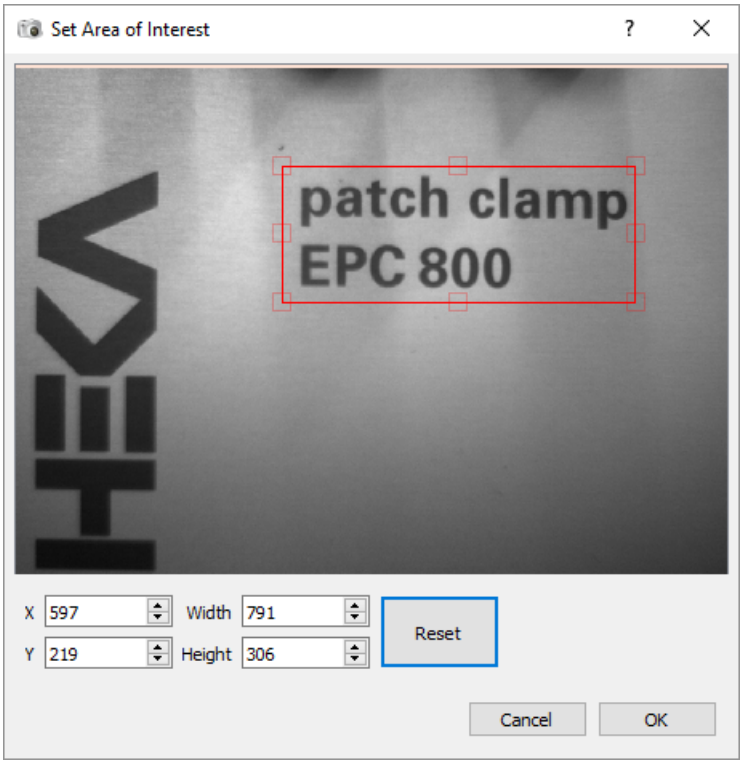


Figure 6.4: *Area of Interest* selection.

After you defined your area of interest (AOI) you confirm the selection by clicking on the *OK* button. The Set

Area of Interest dialog closes and the area of interest is shown in your Image window.

Figure 6.5: The defined *Area of Interest* is displayed in the Image Window.

Reset: Resets all *Area of Interest* settings and restores the full view image.

6.1.10 Live View (J)

Starts and stops the live acquisition of the camera. In "Live Mode" the camera is running with its maximal speed.

6.1.11 Snapshot (K)

A click to this button acquires a single image (`YYYY-MM-DD-000.tiff`). The path is defined in the *Imaging Configuration* (6.6 on page 28).

6.2 PGF Primer

Imaging related information about wavelength, exposure times and repetition rates can be automatically integrated in already existing Pulse Generator sequences. All imaging related manipulation of the sequences should be done through the *PGF Primer*. Once the desired imaging *Parameters* are found, the user can integrate them into multiple Pulse Generator sequences.

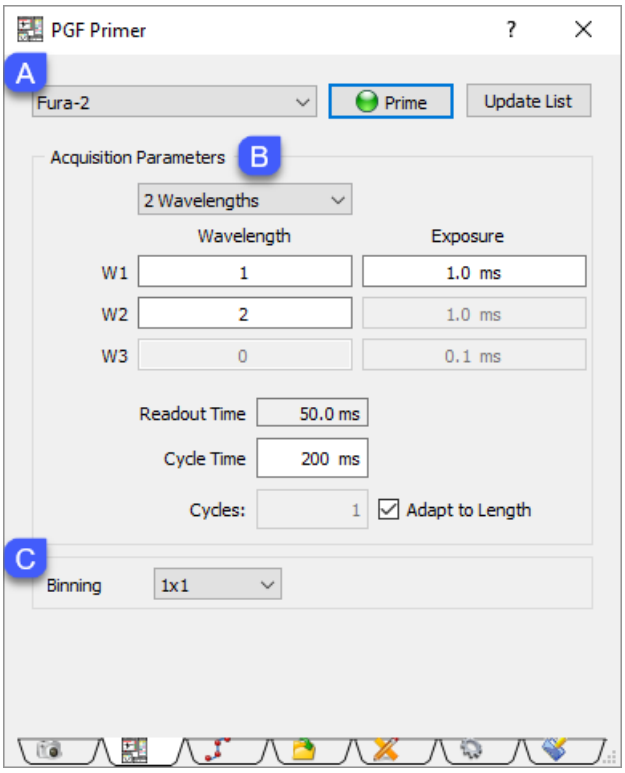


Figure 6.6: `PGF Primer` settings of the Image Control Window of SmartLUX.

A detailed explanation how these settings integrate into the PGF sequence is provided in chapter 7 on page 31.

6.2.1 PGF Control (A)

In this section you have access to the loaded PGF sequence pool to define the define the sequence(s) for the imaging experiment. Further, you control when to transfer (prime) the settings to the selected PGF sequence.

PGF Listing: A list which contains all Pulse Generator sequences currently loaded in Patchmaster. You can select a specific sequence to work on. In case the selected sequence does already contain imaging related parameters (e.g. from previous experiments) the imaging settings of this tab will be updated automatically.

Prime: Transfers the imaging related parameters to the selected Pulse Generator sequence.

Update List: Reads the complete Pulse Generator pool from Patchmaster and updates the *PGF Listing*.

6.2.2 Acquisition Parameters (B)

In this section one defines all imaging parameters like wavelenght, exposure time....

Number of Wavelengths: You have to specify how many different excitation wavelengths (up to three: W1, W2, W3) you want to use in a single imaging cycle.

Wavelength: Specify the wavelength by either entering the wavelength in [nm] or the filter position number.

Note: *Light sources like DeltaRAM and Polychrome need a wavelength setting in [nm] whereas DG-4/5 or Lambda 10 need the filter position set there.*

Exposure [ms]: Enter the exposure times for the different wavelength.

Readout Time [ms]: Displays the approximate camera read out time for one image (display field). This value is specified by the camera and cannot be modified by the user. However, depending on the camera model you are using it can be possible to reduce this value by increasing the binning size.

Cycle Time [ms]: Total duration of one imaging cycle. One cycle can contain 1 to 3 individual image acquisitions.

Cycles: Enter the number of cycles (iterations) per Sweep. In case the option *Adapt to Length* (see below) is selected, this field displays the maximal number of repetitions after the priming process which fits into one Sweep.

Adapt to Length: This option allows to repeat the parent imaging cycle as many times until the duration of the longest Sweep in the Pulse Generator sequence is reached.

6.2.3 Binning (C)

Select the binning size for the image readout. A higher binning size can increase the readout speed which allows higher image acquisition rates.

6.3 ROI Manager

When the *ROI Manager* tab is selected the user can draw regions of interest (ROIs) into the image displayed in the Image Window. The drawn ROIs are listed in the main field of the tab.

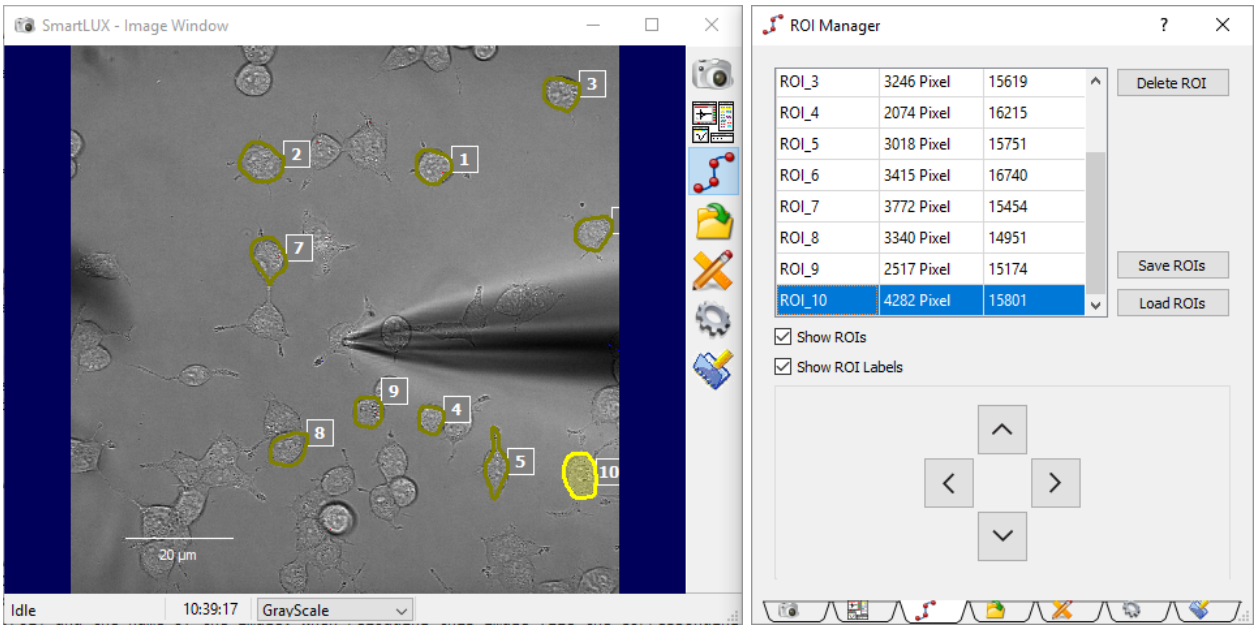


Figure 6.7: Image Window showing cells marked with regions of interest (ROIs) and the `ROI Manager` tab of SmartLUX.

List of ROIs: All ROIs drawn in the Image Window are listed in the `ROI Manager`. The nomenclature is fixed and starts with "ROI_1" increasing the index number until "10" which is the maximum number of ROIs which can be handled at once. For each ROI the total number of covered pixels and the mean intensity value is displyed. The selected ROI from the list is shown in bright yellow color in the Image Window.

Delete ROI: Deletes the selected ROI from the list.

Save ROIs: Saves the current set of ROIs to a separate file with extension `*.roi`. In case an image acquisition is started from PATCHMASTER the ROIs, which belong to this image set, are automatically stored to a file with extension `*.roi` and the name of the image. When reloading this image file the corresponding set of ROIs is also loaded.

Load ROIs: Loads a set of ROIs from a `*.roi` file.

Show ROIs: When activated the ROIs are shown as overlay to the image.

Show ROIs Labels: When activated the index number of the ROIs are displayed with the ROIs itself.

6.4 Image File Selector

The `Image File Selector` tab provides information about the currently loaded images. Further, this dialog can be used to load and display images of previous recordings. It does also allow to watch individual images of your recorded TIFF stack.

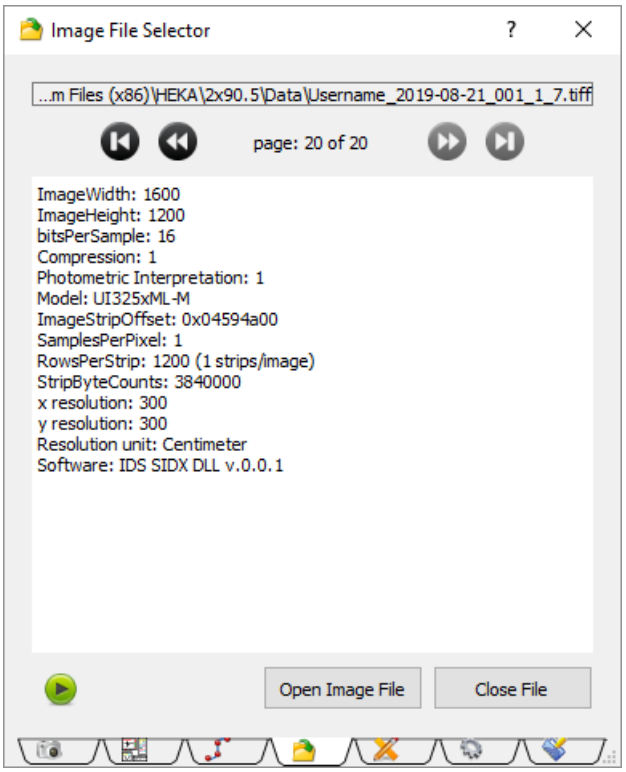


Figure 6.8: `Image File Selector` dialog of SMARTLUX.

Filename: Shows the name and file path of the active image. Hover with the mouse over the text field to see the full path.

Page Selector: In case the image file contains several image frames you can use the left and right arrow buttons to step through the individual frames of the image stack.

Image Parameters: The text box displays the image parameters which are stored with the image file. The information provided here can be very helpful to check for proper image settings.

Play Button: If the image file contains several image frames one can replay them all by clicking on the green play button.

Open Image File: Allows to open an image file.

Close File: Closes the currently loaded image file.

6.5 Calibration Settings

The `Calibration Settings` allow to define and store several *Calibration Sets* for scaling definitions of the image. Once the calibration is set or loaded the scale definition is showned properly in the Image Window.

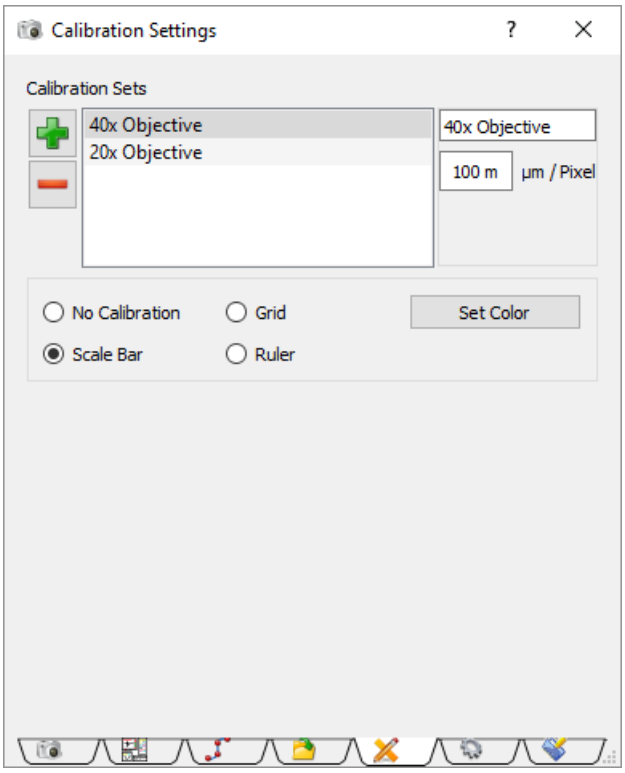


Figure 6.9: `Calibration Settings` dialog of SmartLUX.

Calibration Sets: Press the "+" or "-" sign button to either add or delete a *Calibration Set*. The list of available *Calibration Sets* is displayed in the middle of the dialog. On the right side you can define a name for each *Calibration Set* (select it before changing any names). The last and most important entry box is below the name entry field. There you need to provide the pixel size for the scale calibration. Be aware of the used optics in your experimental setup regarding the calculation of this value.

Scale Options:

- No Calibration: No calibration is displayed in the Image Window.
- Grid: The calibration settings are used to draw a grid in the Image Window.
- Scale Bar: A scale bar is displayed in the Image Window.
- Ruler: A ruler is displayed in the Image Window.
- Set Color: Opens a color selection dialog to define the color scheme of the scaling type.

6.6 Imaging Configuration

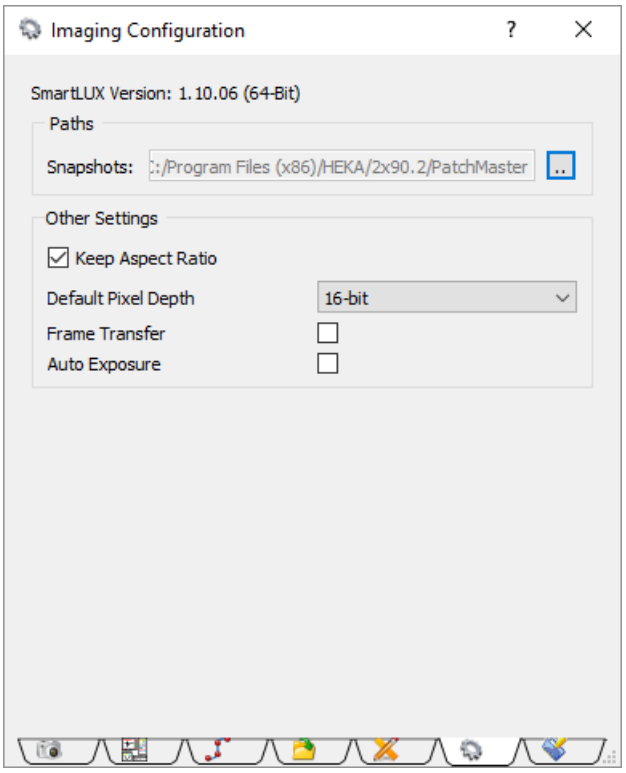


Figure 6.10: `Imaging Configuration` dialog of SmartLUX.

Paths:

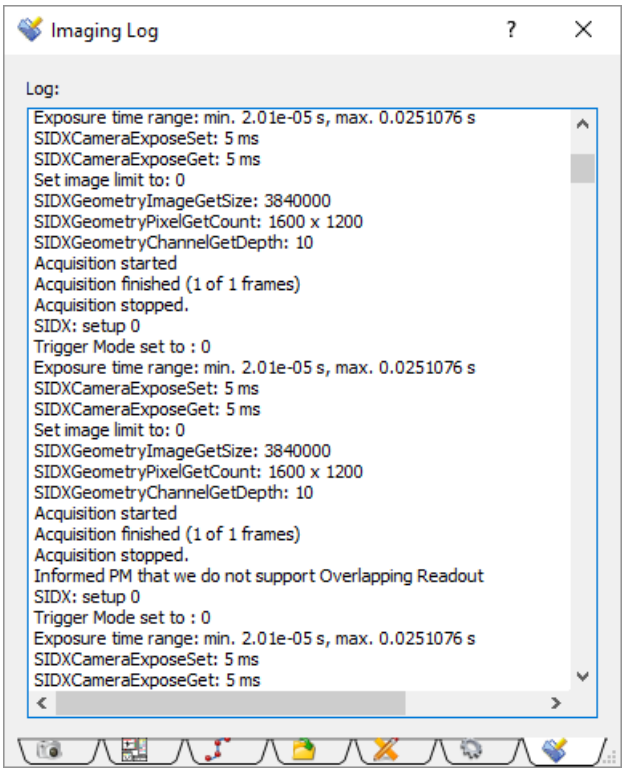
- Snapshots: Shows the path to the folder in which all snapshots will be stored. Press the button on the right in order to change the folder.

  Note: *Images acquired with the Pulse Generator are stored in the same folder as the* PatchMaster *data file.*

Other Settings:

- Show Tab Bar: If activated the *Tab Bar* is shown in the foot line.
- Keep Aspect Ratio: If activated the aspect ratio of the camera sensor is kept proportional when enlarging the window.
- Default Pixel depth: Select the dynamic range of the digitalization (8, 12, 14 or 16 bit).

6.7 Imaging Log



Log File Text: Protocols the communication between SmartLUX and Patchmaster. This information is used to facilitate the troubleshooting.

7. Segment Pattern

Once the settings are made in the `PGF Primer` of SmartLUX and the *Prime* button was executed one can check the modified Pulse Generator sequence. The priming process added some more channels in addition to Channel 1 (for electrophysiology):

- An output channel for the camera trigger (here: Dig-0)
- An output channel for controlling the wavelength of the e.g. monochromator (here: DA-1)
- Input channels for each wavelength (here: Image_W1, Image_W2, Image_W3)

Let us have a look for the content of each individual channel starting with the channel entries of *Channel 1*. *Channel 1* we defined the stimulation template for the electrophysiology recording. This is the initial first step which is essential before using SmartLUX to prime the PGF sequence with further imaging settings. Here, *Stim-1* was defined as the output channel and *Imon-1* as the input channel. We have one segment with a total length of "1000" ms. Of course your real electrophysiological experiment might be a bit more complex.

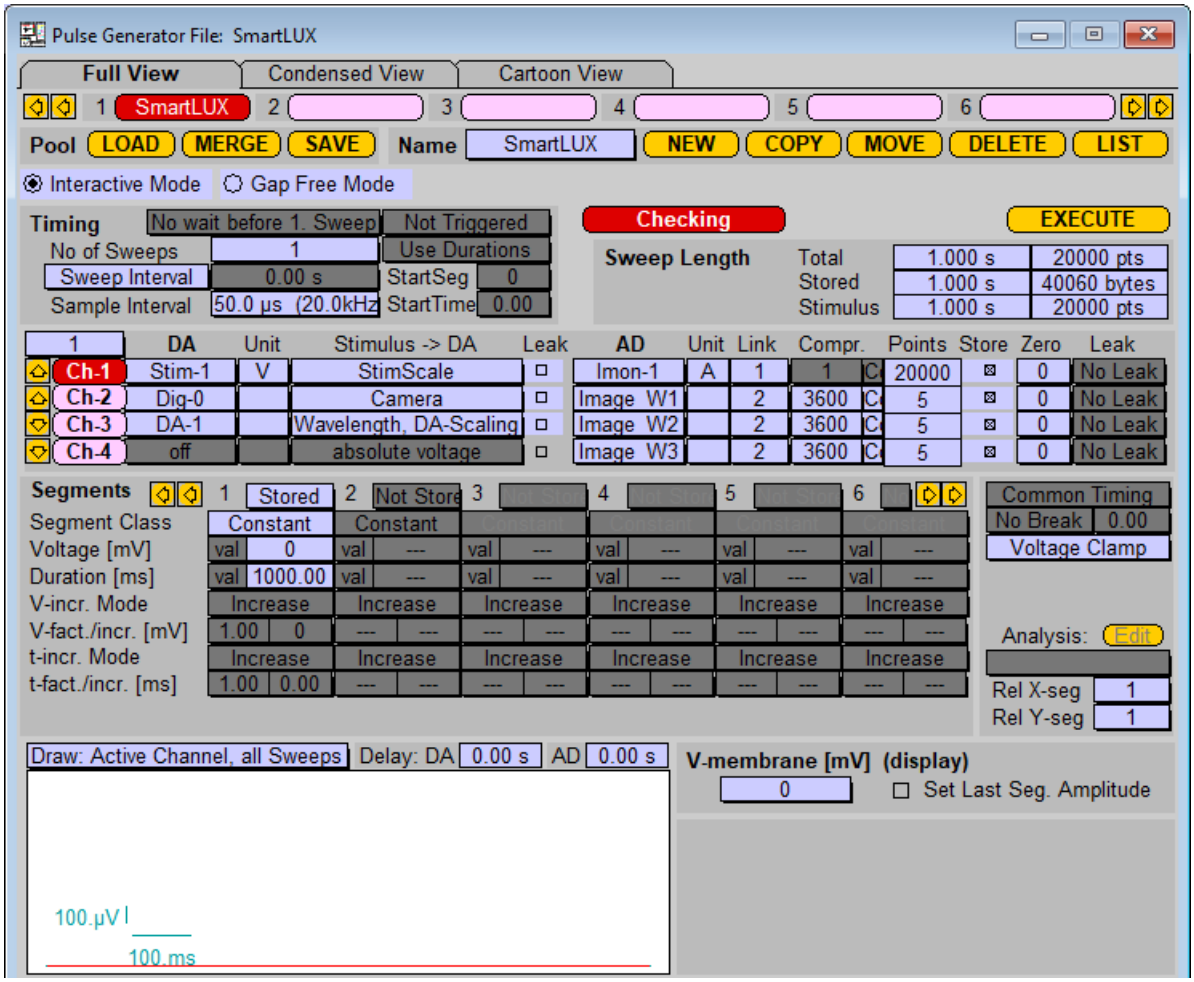


Figure 7.1: *Channel 1* of the primed *PGF sequence*.

The second input channel was set by SmartLUX. As output channel the digital output *Dig-0* was set. It is also labeled with "Camera" to indicate the camera trigger channel. This output channel was defined in the `Imaging`

dialog of the excitation wavelength device. In addition several input channels were defined which are labeled "Image_W1", "Image_W2" and "Image_W3" because we used three wavelengths and set one ROI. All of these input channels are linked to *Channel 2* ("Link" set to "2") and are compressed yielding 5 data points (five images for each wavelength because we have five cycles). One can also see in the cartoon showing the stimulation that we have 15 camera triggers (five for each wavelength).

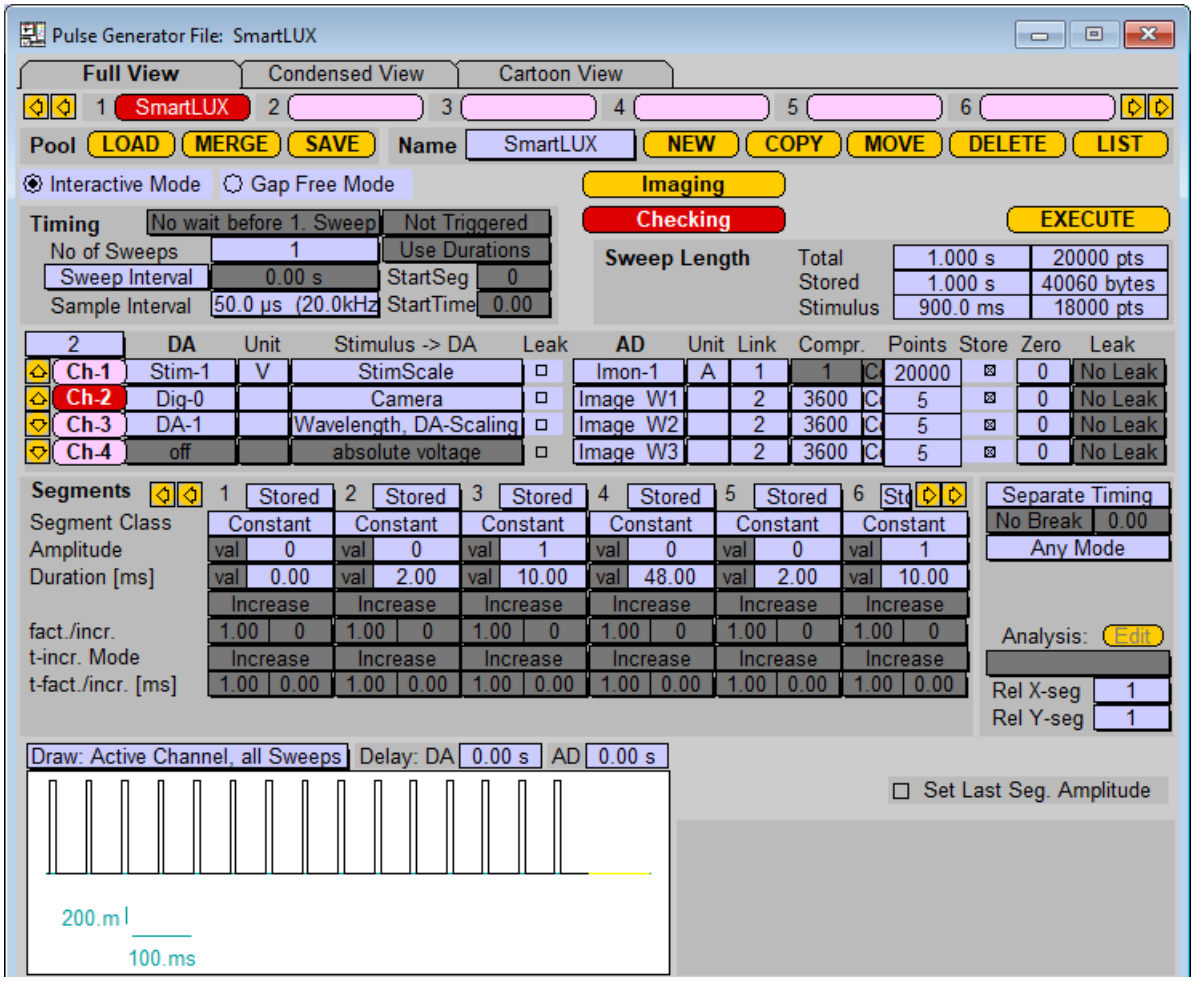


Figure 7.2: *Channel 2* of the primed *PGF sequence*.

Note: *The number of input channels depend on the number of set regions of interest (ROI) and the number of wavelengths. The number of ROIs is multiplied with the number of wavelengths which can result up to 30 wavelength traces (10 ROIs x 3 Wavelengths). Further, consider that a wavelenght trace is only created if at least one ROI was defined before the priming process.*

Channel 3 controls our light source. Therefore, the set output channel *DA-1* (also defined in the `Imaging` setting dialog of the excitation light source in PATCHMASTER) is labeled with "Wavelength". The cartoon does show again the wavelength excitation pattern of our example. We have five imaging cycles each of them with three wavelengths (340, 380 and 440 nm). No input channel is defined here.

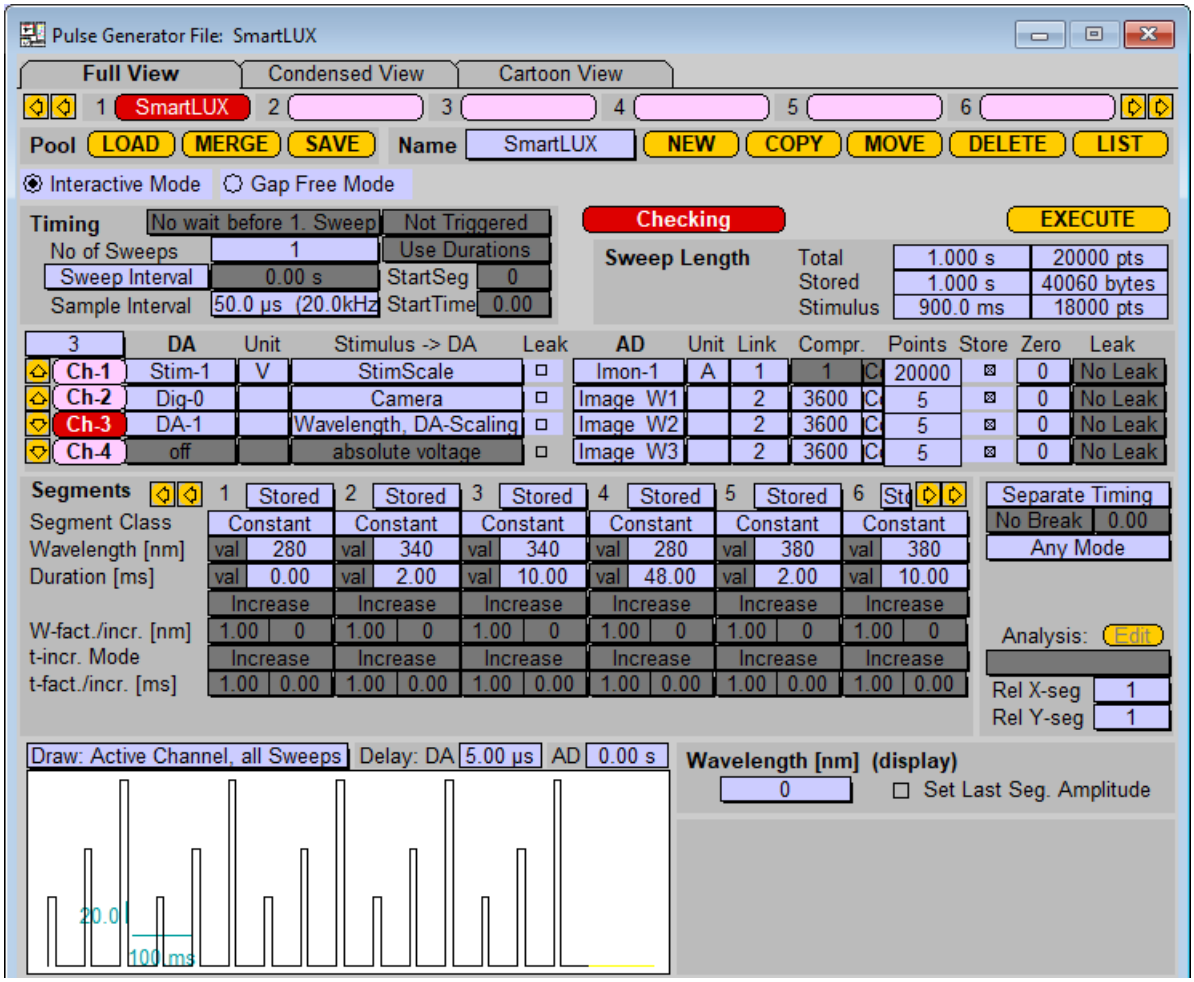


Figure 7.3: *Channel 3* of the primed *PGF sequence*.

In the next chapter we will provide an insight into the segment pattern which is created by SmartLUX in the stimulation sequence.

7.1 Understanding the Segment Pattern

For a better understanding of the inserted channels and the segment patterns through the priming process we split up the segment pattern for each channel. Let us start with Channel 2 which defines the segment pattern for the camera triggering.

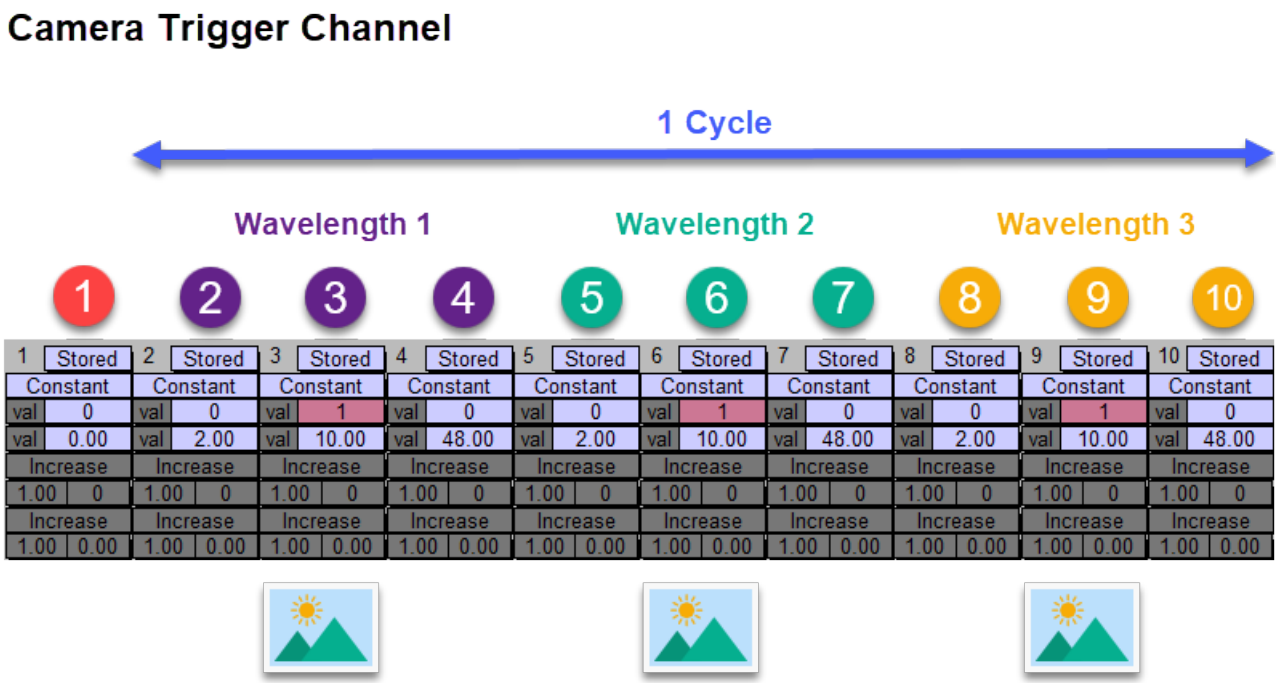


Figure 7.4: Segment Pattern for the Camera Trigger Channel (here: Channel 2). One image is taken for each of the three selected wavelengths.

- Segment 1: Shifting Segment
- Segment 2,5,8: Dead Time (Defined by the light source)
- Segment 3,6,9: Camera Trigger Output
- Segment 4,7,10: Gap/Fill Time (Readout Time - Dead Time)

The segment pattern does always contain a first segment which is used to shift the whole camera and wavelength triggers in respect to the electrophysiological channel. This first segment does only appear once in front of the first cycle. The "Cycle" itself does always consist of nine repeated segments (segments 2-10). For each wavelength three segments are defined. A cycle does always start with the *Dead Time* which would be a waiting time until the light source has set the proper excitation wavelength (segments 2,5,8). The value is variable depending on the used device. The second segment contains the trigger for the camera to acquire an image (segments 3,6,9). The last segment of the triplet is for the gap. This means the segment length is defined by the readout time of the camera minus the *Dead Time*. If we sum that up we would have 60 ms total length for each segment resulting in a total cycle length of 180 ms.

> ***Note:*** *The number of segments is kept constant even if one uses only one or two wavelengths. In such a case the segment duration is set to "0" for the unused segments.*

The last segment of each cycle, here it would be segment 10, is a bit special. It is used to time the distance between each cycle or to store the residual sweep time to match the number of cycles and the sweep length.

Examples of the timing are given below in the table:

Table 7.1: Sample calculation for segment durations of one cycle depending on *Cycle Time* and number of cycles. *Adapt to Length* is activated, therefore the number of cycles tries to fill the full sweep length of 1000 ms. *Dead Time* = 2 ms, *Readout Time* = 50 ms, *Excitation Time* = 10 ms, 3 wavelengths.

	Cycle Time		
Segment No.	180 ms	300 ms	500 ms
2	2	2	2
3	10	10	10
4	48	48	48
5	2	2	2
6	10	10	10
7	48	48	48
8	2	2	2
9	10	10	10
10	48	168	368
# Cycles	5	3	2
Total Time = *Cycle Time* x *Cycles*	900 ms	900 ms	1000 ms

Note: *If Adapt to Length is inactive the minimum Cycle Time will be calculated.*

The timing pattern is identical for the wavelength channel (here: Channel 3). Instead of setting a camera trigger the exciation wavelength is set. This process already starts in the *Dead Time* segment to get the filter wheel (or similar) set to the wavelength to guarantee proper excitation time in the following segment.

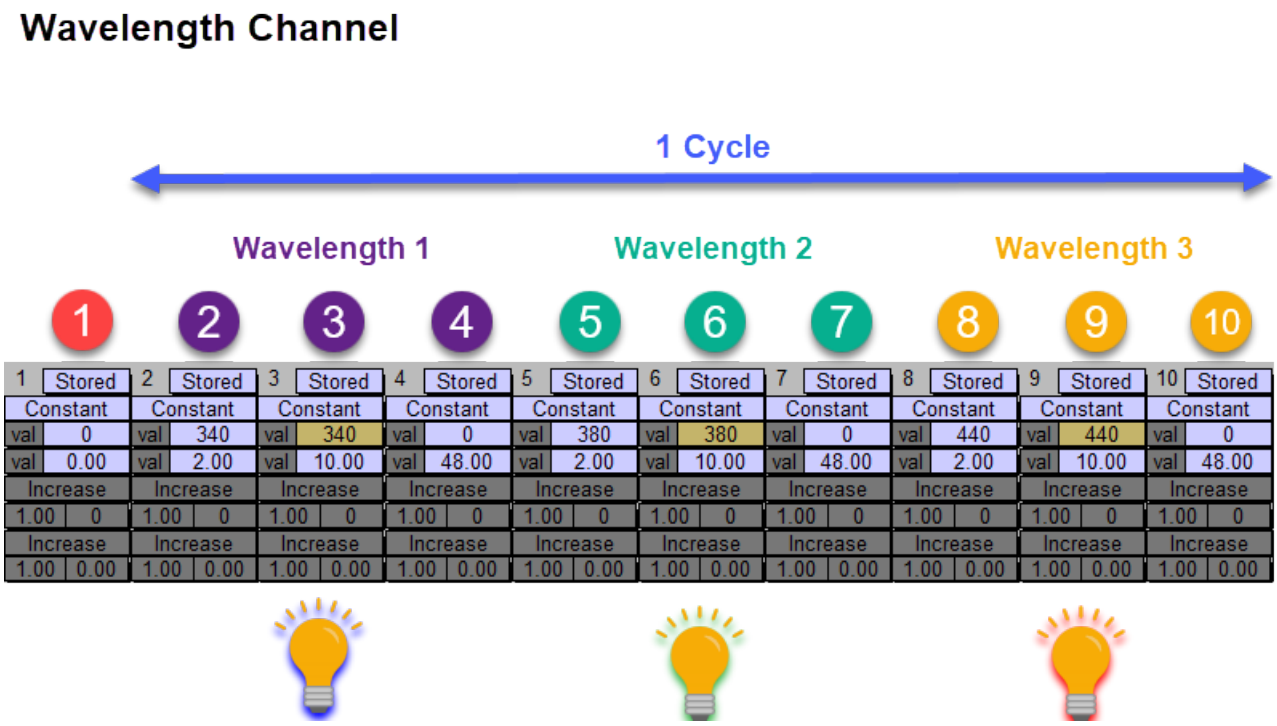


Figure 7.5: Segment Pattern for the Wavelength Channel (here: Channel 3). Each excitation wavelength is set during the *Dead Time* segments (2,5,8) and the real excitation time is defined in segments 3,6 and 9.

8. Tutorial

8.1 Ratio Imaging with SmartLUX

The HEKA amplifiers/potentiostats of the EPC 10 USB or PG 618 USB series in combination with the software packages PATCHMASTER or POTMASTER and their SMARTLUX extension, support synchronized image acquisition. Quanitative fluorescence imaging is done best with a ratiometric fluorescence dye. Here in our example, we are using the dye Fura-2 for measuring the intracellular calcium concentration of a single cell. HEKA provides a set of files to help the user performing a Ratio Imaging experiment.

The protocol "TimeLapseImaging" controls the seamless switching between repetitive ratio acquisition and other acquisition sequences which are executed on user command.

8.1.1 Loading the RatioImaging Set Files

8.1.2 Adjustments to the Configuration

8.1.2.1 Imaging Hardware

In case you have not used the SmartLUX system with your setup before, you may configure the software with respect to the connected hardware. Open the Imaging window (compare **??** on page ??) and check the parameters for

- Background parameters should be zero (at least for an initial test).
- Choose an appropriate *Dead Time*. Note in our experiment we are using an LED light source which can be digitally controlled (e.g. DG-4/DG-5 or Lambda 10). Hence we use such a configuration with a small *Dead Time* of 1 ms.
- For the Ratio Imaging experiment we check *Ratio* as *Virtual Traces to create*.
- As *Camera Trigger* we use one of the free DA channels (because the digital channels are already in use for controlling the light source.)

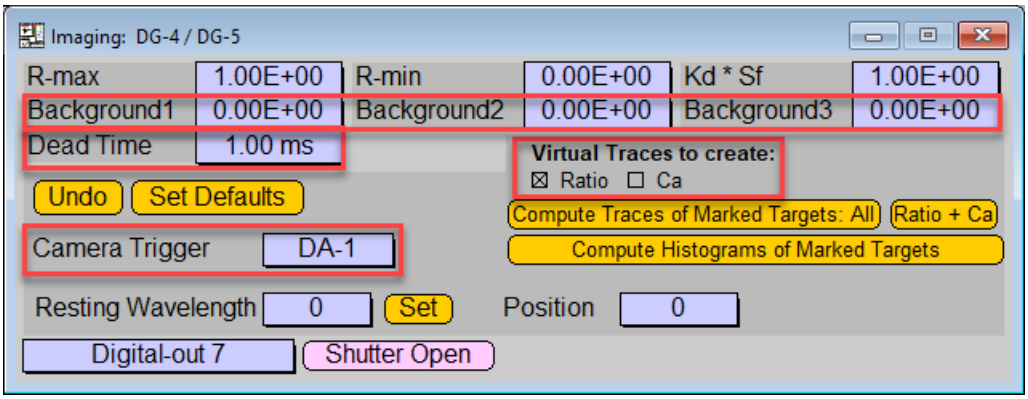


Figure 8.1: `Imaging` settings dialog of PATCHMASTER.

8.1.2.2 Trace Index

The Analysis method Fura-2 has been setup for using the Trace Assignment feature. Here, the fluorescence of wavelength 1 is calculated as mean of trace Image_W1_1.

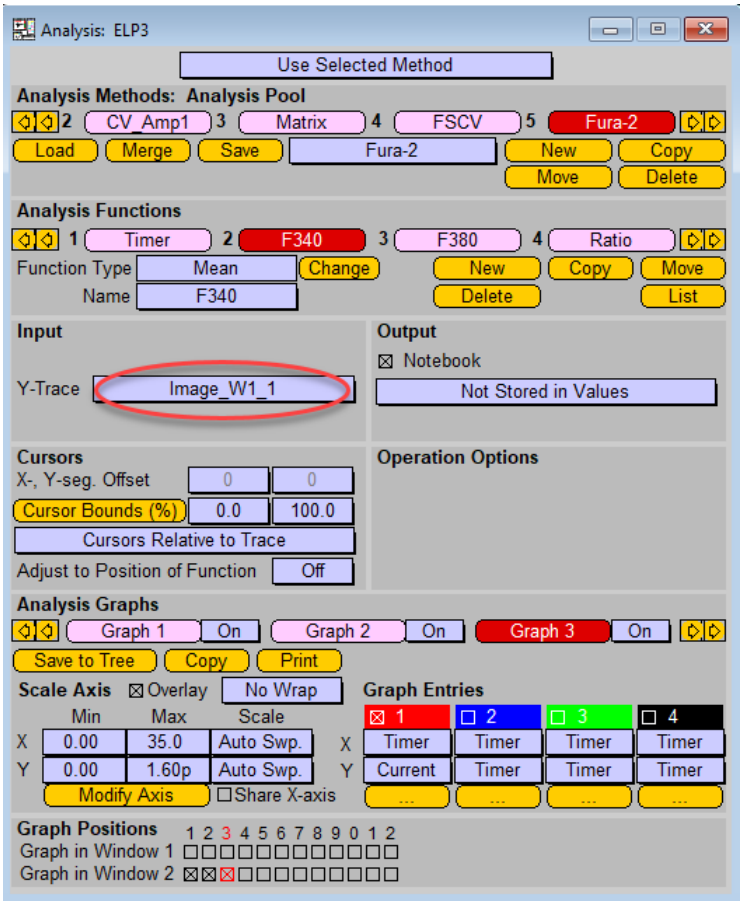


Figure 8.2: text

That means, each created traces as a specific assingment and can be addressed independently of the order or number of acquired traces.

If you want to load this example to your setup, please make sure that you have a TraceAssignment created and that at least the traces Image_W1_1, Image_W2_1, and Image_R_1 are defined.

Below you see a typical trace assignment for an ElproScan System. The traces 15 to 21 address the Imaging extension.

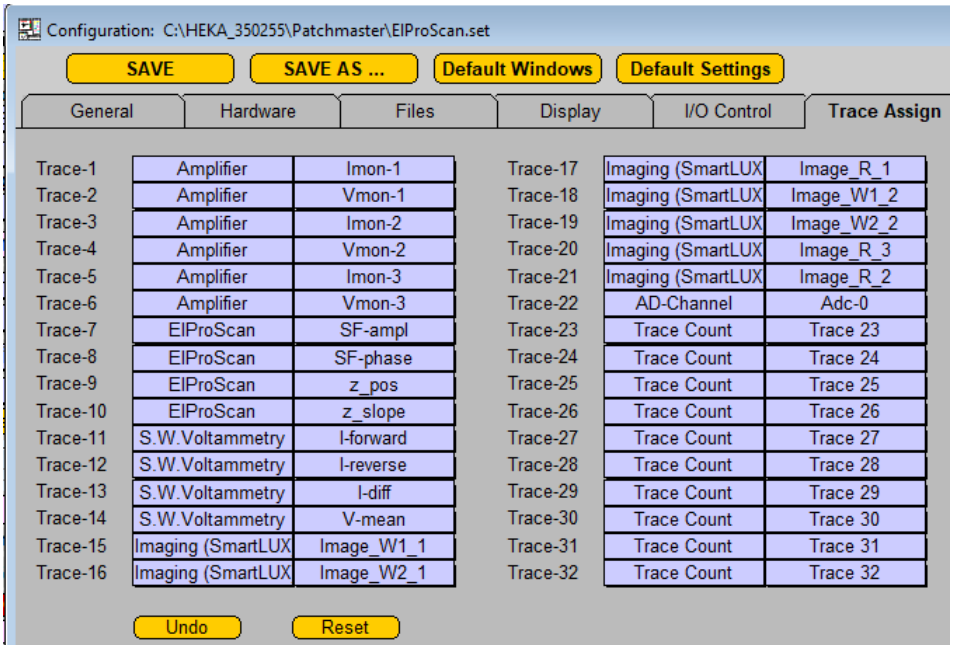


Figure 8.3: text

8.1.2.3 Merging Files

- To the pool of Pulse Generator sequences (open the editor by pressing F8), merge the file “RatioImaging.pgf”. The sequences “Fura-2” and ”Fura-2-Burst” will be added to the pool. Save the pool.
- To the pool of Analysis methods (open the editor by pressing F7), merge the file “RatioImaging.onl”. The method “RatioImaging” will be added to the pool. Save the pool.
- To the pool of Protocols (open the editor by pressing F9), merge the file “RatioImaging.pro”. The protocol “TimeLapseImaging” will be added to the pool. Save the pool.

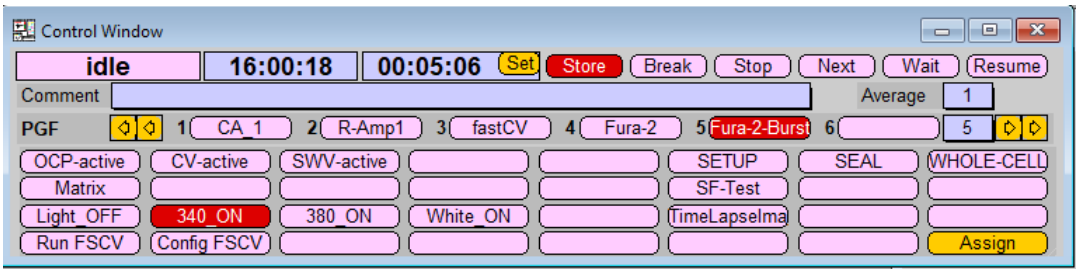


Figure 8.4:

8.1.3 Searching for a Cell and Testing for Exposure Time

Turn on the transmission light source and start the LIVE View in the CameraSetting window (6). Focus on the cells and move a cell of interest into the middle of the camera’s field of view. Now, turn off the transmission light and turn on the Fluorescence Excitation light. Here in this example execute the protocol 380_ON, which turns on the 380nm LED of the connected fluorescence excitation light source. Now, check if the cell of interest shows a fluorescence signal and adjust the focus if necessary.

You may apply optimization of the fluoresence image acquisition with respect to file size, spatial resolution, acquisition speed, and fluorescence intensity.

Adjust the exposure time or the light intensity for a good image quality. Do the same test for the 340nm wavelength. You have now good estimates for appropriate exposure times.

8.1.4 Adjusting the Image Size

8.1.4.1 Binning

You may increase the binning factor in case you don't need the highest possible spatial resolution. Especially, if you are interested in the mean fluoresence intensity within a ROI, the binning factor can be increased without loosing significant information. A larger binning factor has the following advantages:

- The read out time of the camera is shorter and therefore higher frame rates can be reached.
- The size of a single image is smaller.
- If the camera supports on-chip binning, the fluorescence intensity increases, because the photons of several pixels is binned. Note that some cameras correct for the number of source pixels, which contribute to the binned pixel. In this case the intensity stays unchanged, but the signal is less noisy.

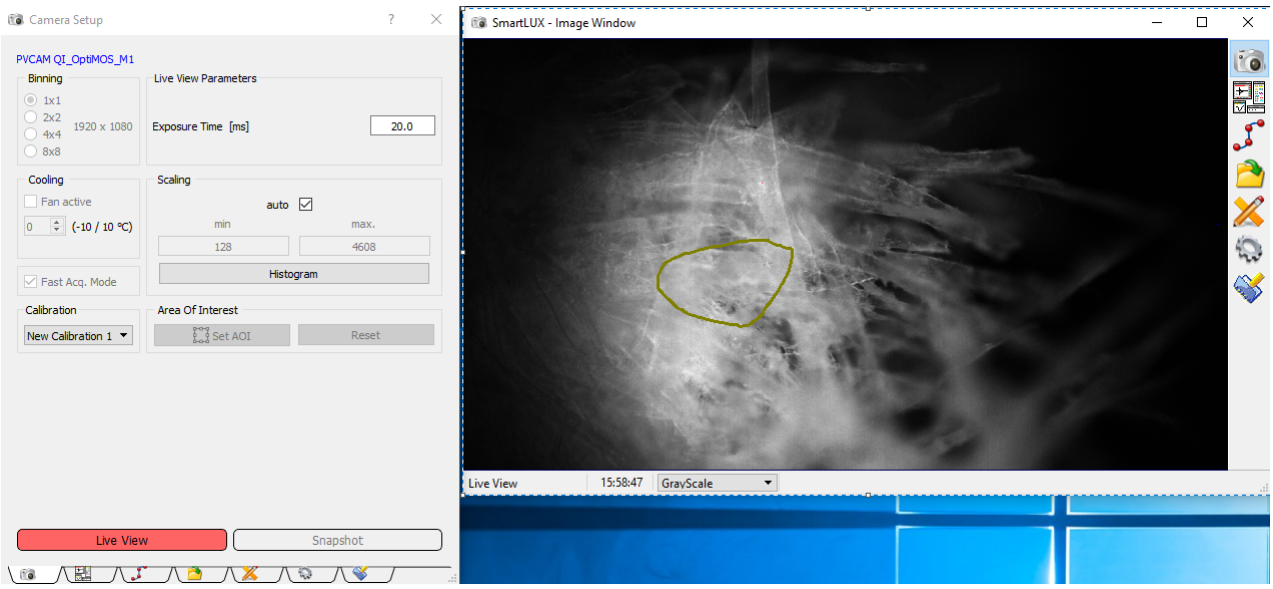
8.1.4.2 Area of Interest

Some cameras allow to read out only a certain area from the camera chip. This area is called Area of Interest (AOI). Acquiring only a part of the camera chip has the following advantages:

- The size of the store image is smaller.
- The read out time is smaller, because a smaller number of lines is read out.

8.1.5 Setting up the Acquisition

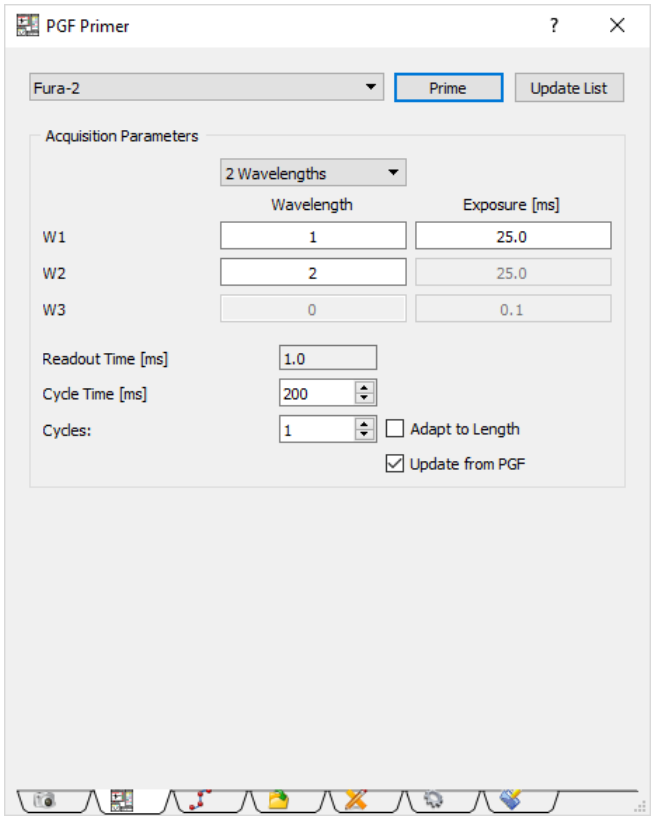
Before starting the experiment, please switch to the ROI pane and draw a region of interest (ROI) over the cell of interest.



In a next step, you have to add the imaging information to your pgf sequences. In our example, we will use the Fura-2 sequence as acquisition sequence, which will run in a time lapse mode. This means, we will acquire images at a low repetition rate (e.g. 1Hz) and plot the fluorescence intensity data versus time.

- Select the Fura-2 sequence from the list at the top of the pane. If you can't find the sequence in the list, click Update List. For a ratio imaging experiment using fura-2, we will need two wavelength.
- Depending on the light source used, enter the filter position numbers or wavelength for wavelength 1 and 2. Note that the ratio is calculated as fluorescence at wavelength 1 divided by fluorescence at wavelength 2.

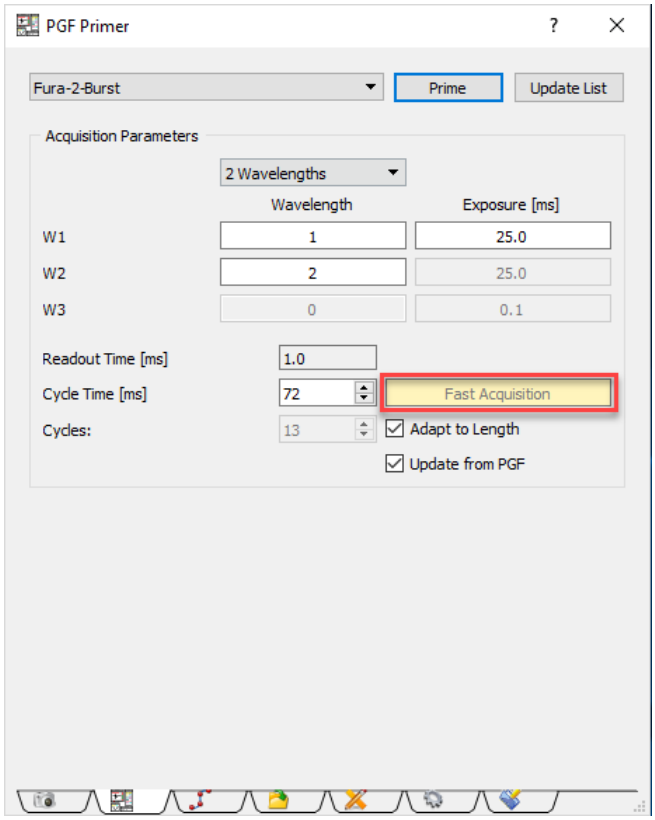
- Now, enter the exposure time, which you have estimated before.
- Enter a cycle time of 200ms.
- Enter 1 cycle.
- Deselect the option Adapt to Length.
- Now, press the Prime button at the top of the pane and all information is added to the Fura-2 pgf sequence.



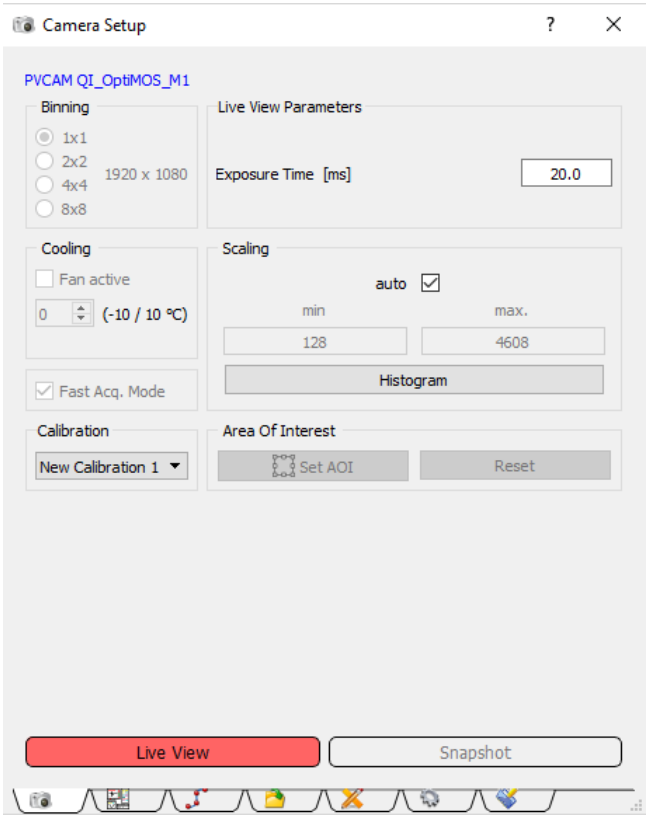
You should repeat the priming process for all acquisition sequences you are planning to execute during the experiment. Here in our example, we are priming the sequence Fure-2-Burst as well. Hence, we repeat the precedure described above with this seqeunce.

However, we will

- Enter a small Cycle Time. E.g. if you enter 1ms, the system will calculate and set the Cycle Time to the fastest possible.
- Check the Option Adapt to wavelength



In our example, the fastest Cycle Time is 72ms and we will execute 13 Cycles within a single sweep. Note: The button Fast Acquisition shows up. This means the acqusition rate is so fast that there is not enough time to display and analyze the images. The images will be stored and the use has to analyze them offline.



8.1.6 Background Subtraction

SmartLUX provides the feature to subtract a constant fluorescence intensity from the fluorescence values of the corresponding wavelength before caclulation of the ratio.

We suggest to perform a test image acquisition before starting the experiment. From this test acquisition, you can check if the exposure times and signal quality is acceptable and you can read the background fluorescence values.

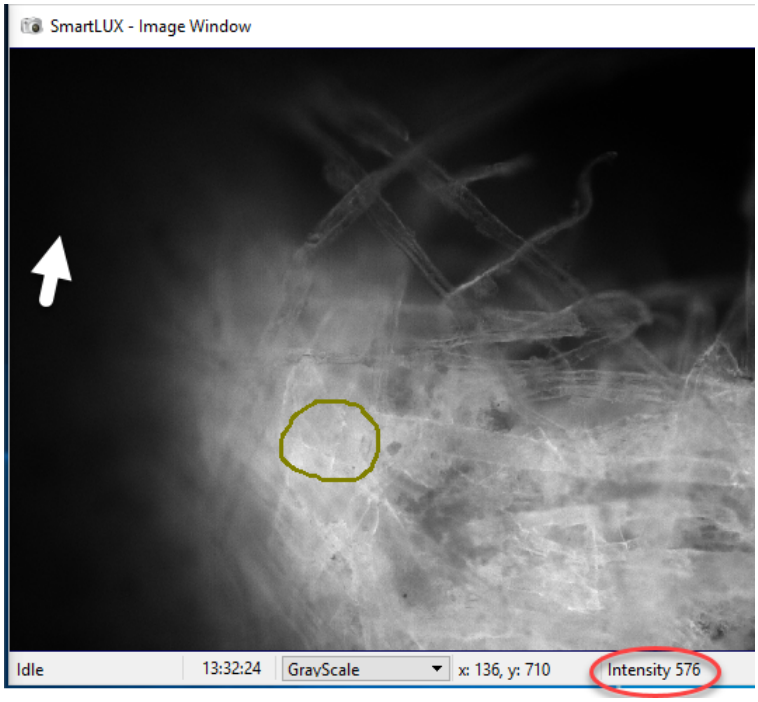
Please start the pgf sequence Fura-2 and let the system acquire a few image pairs. Then press Stop to terminate the acquisition.

In SmartLUX, the images of the test acquisition are still opened. Please switch to the Image File Selector (**??**)  pane and you can step through the images of the recently acquired image stack. The last image in this stack was acquired at wavelength W2, which is 380nm in our example.

After you have selected a background value for the last image (wavelength 380nm), you switch to the preceeding image by clicking to the left arrow  and repeat the background analysis for this image as well.

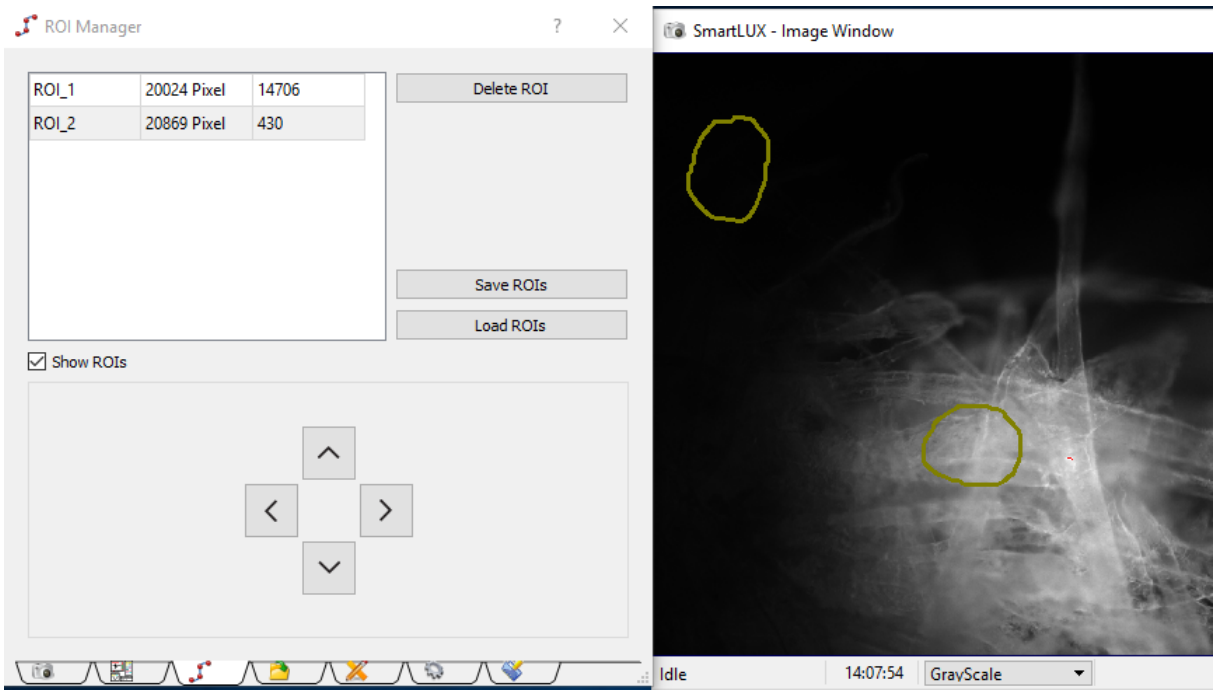
Here we are presenting two methods on how to read the background value from an image:

- Use the cursor tip intensity reading. If you move the mouse cursor over the image, the position of the cursor tip as well as the intensity reading will be displyed in the footer of the image.



  You may read and pick a typical background intensity as background value for this image.

- Use a ROI for background caculation. Switch to the ROI Manager pane  and draw another ROI in an area of background to the image.

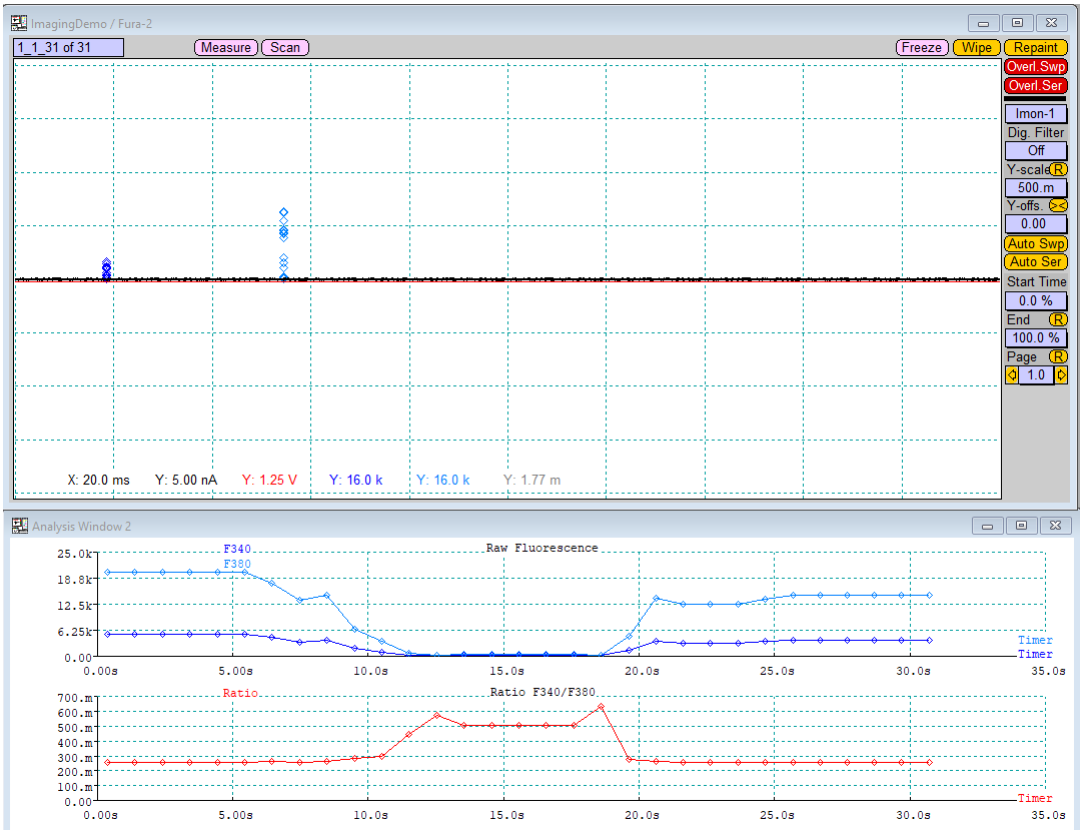


The area and the average fluorescence intensity of the ROI will be displayed in the table of ROIs. This method has the advantage that the program averages several pixels. You may delete this ROI after background measurement has been done for all wavelength. Otherwise separate traces will be created in PATCHMASTER and this ROI will be handled as any other ROI.

8.1.7 Running the Ratio Imaging Experiment

Before starting the experiment, please make sure that the Analysis Window 2 is opened and visible on the screen.

Finally, start the protocol TimeLapseImaging. Every second a pair of images will be acquired. Current/Voltage as well as the mean fluorescence intensities are plotted in the Oscillocope window. In addition, the fluorescence as well as the ratio is displayed versus time in the Analysis Window 2.

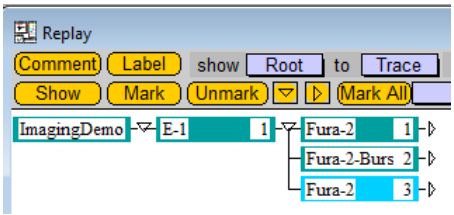


Pressing ”1” on your keyboard will stop the acquisition, execute the sequence Fura-2-Burst, and finally start the Fura-2 sequence again.

Note: Protocol can be extended with the Freeze feature...

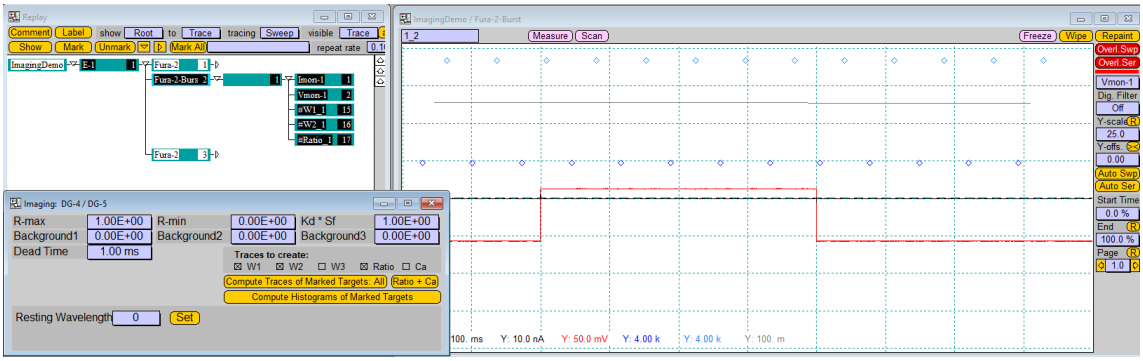
In this example we are stopping the experiment after short while.

In the Replay Window we can see that there are three sequences acquired. We can show the complete experiment by replaying the entire group (here E-1).



The sequence Fura-2-Burst was acquired in FastAcquisition mode. Hence no fluorescence data have been transferred to PATCHMASTER/POTMASTER yet.

We mark the sequence, open the Imaging Window, and press ”Compute Traces of Marked Targets:All”



In the oscilloscope you will see three additional traces for WL1, WL2, and Ratio of the first ROI.

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