**Example data set:** MitoMemPot1.zip and MitoMemPot1_ICC.zip

**Pipelines used:** Mitochondrial membrane potential measurement (TMRM/PMPI) with post-hoc ICC-based ROI drawing

## QUICK START GUIDE

1. Unzip contents of MitoMemPot1.zip and MitoMemPot1_ICC.zip onto your hard drive in the same folder.
2. Open the recording using the File/"Open Image Series/Measurement" or the 🔷 toolbar button. In the Open Image/Measurement dialog select Elements ND (*.nd2) as file type. Order files by their names and select all numbered nd2 files. Press Open. Alternatively use multiple file selection in the Windows Explorer and drag-and-drop on the Image Analyst MKII. *Note: for this experiment it is suggested to use the internal loader, which is the default, and can be selected in the File/"Set Folder Locations and Default Open Method".*
3. Tag this Multi-Dimensional Open dialog as #1 in its top right corner.
4. In the Multi-Dimensional Open dialog, use the "File Order" tab to verify that the files are in an incrementing order.
5. Switch back to the "Open" tab and select position 11. *Note: other positions can be also selected, but #11 is demonstrated below).*
6. Open similarly the icc.nd2 file and tag the new dialog as #2.
7. Set the first Multi-Dimensional Open dialog as default by clicking ⚙ and then "Make this Multi-Dimensional Open dialog the default for Pipeline Processing".
8. Select the "Mitochondrial membrane potential measurement (TMRM/PMPI) with post-hoc ICC-based ROI drawing" pipeline in the Pipelines/"Intensity Measurements"/Applications main menu point.
9. The default parameters of the pipeline have been configured to work with this particular recording.
10. Press the 🅿 button on the main toolbar or in the bottom of the Multi-Dimensional Open dialog.
11. To calibrate PMPI and fluorescence traces to millivolts, either follow the step-by-step configuration of the "Membrane Potential Calibration Wizard" (below) or open the "calibration configuration.ips" configuration file accompanying the image data files.
12. Press the ▶ button to perform the calibration or the 🔲 button to transfer calibrated potentials to Excel.

## THE EXAMPLE DATA SET

**Sample:** Human non-diabetic primary β-cells in a coverglass bottomed 96-well plate (Corning 4580). Published in Endocrinology 2015 doi:10.1210/en.2015-1552.

**Fluorophores:**

- Recording 1 (files 01-10.nd2): Channel (1): TMRM filter set: 580/14 – 594 – 641/75 (150 ms exposure time)
- Recording 1 (files 01-10.nd2): Channel (2): PMPI filter set: 500/24 – 520 – 542/27 (75 ms; all filters from Semrock)
- Recording 2 (icc.nd2): Channel (1): Alexa555 fluorescence (insulin immunocytochemistry) 543/22 – 562 – 588/21nm
- Recording 2 (icc.nd2): Channel (2): Hoechst 33342 fluorescence (nuclei) 390/40 – 409 – 460/80nm

**Microscope:** Nikon Ti-Eclipse Perfect Focus System, equipped with a Cascade 512B camera and S-Fluor 20× air lens, a Sutter Lambda LB-LS17 Xe-arc light source and 10-3 filter wheels and an ASI MS-2000 linear encoded motorized stage, controlled by NIS Elements 4.0.

**Experimental paradigm:**

1. Baseline: in PM (see protocol) 30 frames
2. Addition #1: 16mM glucose ~ 30min (for positions 11 and 12)

3.  Addition #2: nothing ~ 30min (for positions 11 and 12)
4.  Addition #3: MDC + ASC2 +TTX (see abbreviations in the protocol or in Gerencser et al. in J Physiol. 2012 590:12 2845-71) ~ 20 min
5.  Addition #4: stir 5 frames, the volume is 150 µl now
6.  Addition #5: +10 µl PMK (supplemented with MDC + ASC2 +TTX)
7.  Addition #6: +20 µl PMK
8.  Addition #7: -40 µl  PM +40 µl PMK
9.  Addition #8: -75 µl  PM +75 µl PMK
10. Addition #9: -100 µl  PM + 80 µl PFA in PMK

## THE PIPELINE: MITOCHONDRIAL MEMBRANE POTENTIAL MEASUREMENT (TMRM/PMPI)

This pipeline performs all image processing tasks required for calibration of a TMRM / PMPI (FLIPR plasma membrane potential assay kit) fluorescence time lapse recording to millivolts. In addition, post-hoc immunocytochemistry (ICC) images (showing the same view field after immunofluorescence staining) are used to selectively circle a specific cell type. Processed images and calculated ROIs are passed to the Membrane Potential Calibration Wizard. Using pre-defined configuration, this pipeline is capable of unsupervised processing of multi-stage position mitochondrial membrane potential assays.

The pipeline requires two Multi-Dimensional Open dialogs as input. One containing the live-cell time lapse of TMRM and PMPI fluorescence, tagged as #1 in the top right corner of the dialog. The second dialog (tagged as #2) opens the post-hoc immunocytochemistry, containing a nuclear stain channel and a qualifier stain (e.g. insulin for beta-cells).

The pipeline corrects for misalignment between channels on the frame-by-frame basis, subtracts background, performs spectral unmixing with pre-defined coefficients, and stabilizes the time lapse (registers frames in time). To eliminate background the "Median of pixels below percentile of max projection" with the percentile given at "Background level" is used.

To determine spectral crossbleed coefficients use the Tools/Calculate Crossbleed Correction Factor main menu point, or the Math/Blind Spectral Unmix with NMF function. See more on the layout of the Spectral Unmix Coefficient Matrix in the description of Math/Spectral Unmix.

Then ROIs are generated automatically using the nuclear stain as seeds, following the shape of the maximum intensity projection of the summed PMPI+TMRM image. Therefore if a cell moves during the experiment, the ROI will contain the cell for the whole duration of the recording. The calibration is not affected by the amount of background recorded in the ROI.

The immunofluorescence stain is used the classify ROIs, removing ones with lower than a threshold fluorescence.

Prepared TMRM and PMPI images with immunofluorescence positive ROIs are passed to the Membrane Potential Calibration Wizard. Calibration can be performed by manual configuration of the wizard, or using a previously saved configuration file to provide automation.

The Membrane Potential Calibration Wizard implements the calibration technique published by Gerencser et al. in J Physiol. 2012 590:12 2845-71.

This pipeline has been used to process single beta-cell potentiometric recordings in Endocrinology 2015 doi:10.1210/en.2015-1552

## CONFIGURATION OF THE MEMBRANE POTENTIAL CALIBRATION WIZARD

The following step-by-step configuration of the potentiometric calibration is given for the analysis of the example data set without loading the accompanying calibration configuration file. The experiment was following the standard potentiometric protocol. If you have already loaded the calibration configuration above, the Wizard can be optionally reset by pressing the ☐ button.

1. **Calibration Method** tab:
    1.1. Choose the plasma membrane potential calibration method: "Complete with known kP (K-steps)"
    1.2. Choose the mitochondrial membrane potential calibration method: "Complete"
2. **Input/Output** tab: the input images have been already selected by the pipeline.
3. **Wizard – Data Ranges** tab:
    3.1. Select baseline by pointing the range in one of the graphs on the left, and press the "Select baseline button" or alternatively 1-30 next to the "Select baseline button"
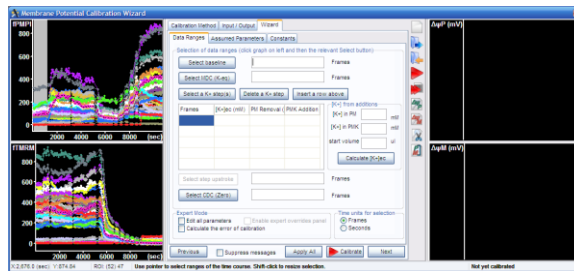


    3.2. Select complete mitochondrial depolarization in the graph, and press the "Select MDC (K-eq)" button (129-161).
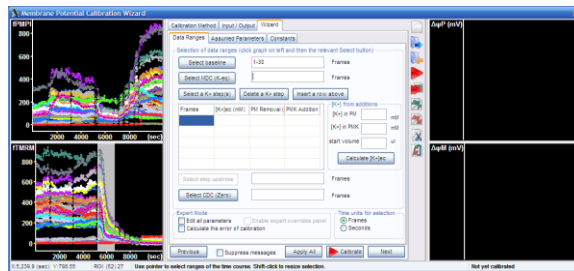


    3.3. Select final complete depolarization in the graph, and press the "Select CDC (zero)" button (187-236).
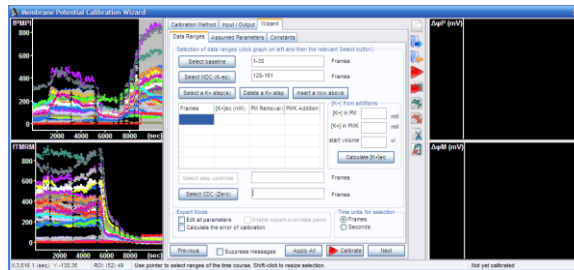
3.4. Select the K$^+$-steps, the five segments visible between the mitochondrial and complete depolarization in the top left graph, and press the "Select a K+ step(s)" button.
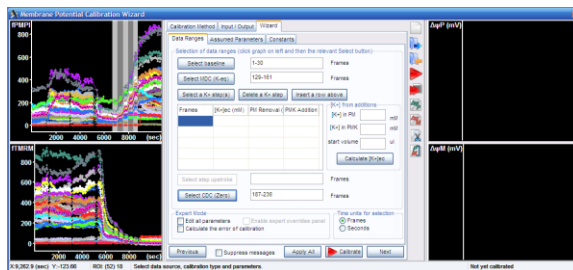


3.5. To calculate [K+] during K-steps, enter the K$^+$ concentration in the potentiometric medium (PM), that was 5.3 mM and in the K-based potentiometric medium (PMK), that was 125.3 mM, and the medium volume (150 µl). The K-steps were performed by first adding 10 µl PMK to the assay well. Enter 10 to the second line and "PMK Addition" column in the K-steps table. This was followed by addition of 20 µl (enter it in the next line), then removal of 40 µl and addition of 40ul (in the next line enter 40 to the "PM Removal" column and 40 to the "PMK Addition" column. Finally 75 µl was removed and 75 µl was added (fifth line of the table). Press the "Calculate [K+]ec button".
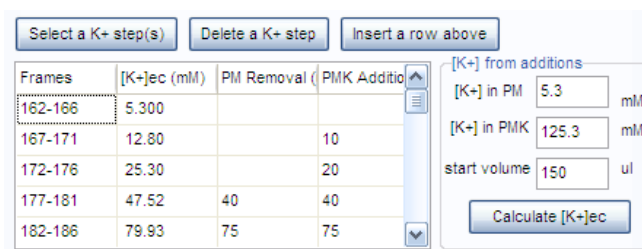


4. **Wizard – Assumed Parameters** tab: No action is required, use the default rate constant for PMPI redistribution $k_P$=0.38 s$^{-1}$, and assume no significant non-K-permeability of the plasma membrane during K-steps by using the default $P_N$=0.
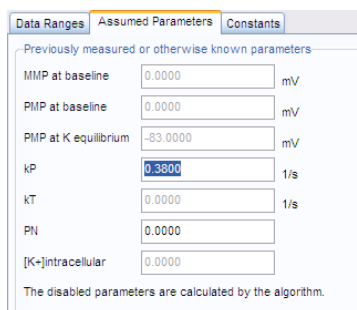


5. **Wizard – Constants** tab: set the cell specific parameters here:
   5.1. VF (mitochondrion:cell volume fraction): use the "Mitochondrion:cell volume fractionator" pipeline to calculate this value using confocal microscopy, assume it based on literature. For the example data set it was measured to be 0.0781 ± 0.0031.
   5.2. VFM (matrix:cell volume fraction): this value affects the results only little (because aR' –see below- largely cancels its effects). The default is 0.8
   5.3. aR' (apparent activity coefficient ratio): use the "Mitochondrial membrane potential assay - measurement of the apparent activity coefficient ratio" pipeline to calculate this value using confocal microscopy. For the example data set it was measured to be 0.360 ± 0.003
   5.4. Leave all other constants at their default values.

6. Press the ▶**Calibrate** button to perform the calibration. ***Note:*** *not all cells in the view field can be calibrated, therefore error messages will appear. Press cancel to see no more messages, or check "Suppress messages" in the bottom of the Calibration Wizard.*
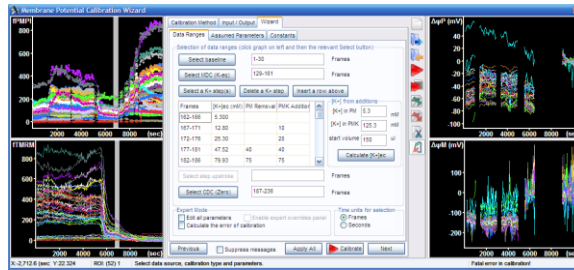


7. **To save results** press 🖼 or right-click / "Copy Plot Data" in the graphs on the graphs on the right.

8. **To explore the results:**

    8.1. Use the 📊 and 📊 buttons to see the regression analysis used to calculate calibration parameters

    8.2. Use the 📊 button to see only the time course preceding the calibration steps.

    8.3. Use the 📊 button to see only those plasma membrane traces that also were successfully calibrated for mitochondrial membrane potential. Press ▶ again to refresh the results.

    8.4. Right-click / "Calculate Mean" to see mean±SE of all calibrated traces. To undo mean calculation, press ▶ again. ***Note:*** *if the mean calculation is performed on the fluorescence traces on the left, then the mean data will be calibrated.*

9. **Quality control of the calibration:**

    9.1. Check "Calculate the error of calibration" in the Data ranges tab within the Wizard tab and press ▶. Now the predicted error of the calibration is shown. ***Note:*** *optionally, check the "Suppress messages" on the bottom.*

    9.2. Check "Edit all parameters". Detailed lists of all parameters for PMPI and TMRM calibration, common calibration constants, and error propagation parameters are shown in tabs appearing on the right of the Wizard tab on the top.

    9.3. In the PMPI parameters, set Quality control by propagated error of baseline to "Yes" and press ▶. Traces with larger predicted error than set at the parameter below disappear now.

    9.4. In the TMRM parameters, set Quality control by propagated error of baseline to "Yes" and press ▶. Traces with larger predicted error than set at the parameter below disappear now.
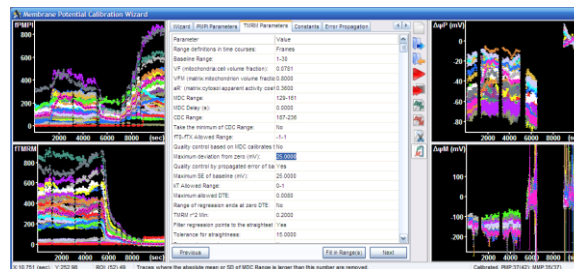


10. **Using the Expert Mode**: When the Edit all parameters checkbox is checked in the Data ranges tab within the Wizard tab, all parameters of the calibration algorithms can be directly accessed in the tabs appearing on the right of the Wizard tab. These can be used alternatively to the Wizards tab to enter any of the parameters. Use the "Fill in Range(s)" button to automatically enter a range from the selection made in the right graphs.

11. **Fine tuning the calibration**

    11.1. Switch to the "Constants" tab, for this the "Edit all parameters" needs to be checked in the "Wizard" tab.

    11.2. To accommodate to the temporal resolution and noise of the recording, adjust the "Differentiation kernel width" to 11 frames. Larger width suppresses noise by providing more smoothing, but also smears fast changes.

11.3. Median filter for baseline, fft0 and fp0: if using the "Take maximum of CDC Range" in the "PMPI Parameters" tab or "Take minimum of CDC Range" in the "TMRM Parameters" use this option to suppress noise affecting minimum and maximum calculations.

12. **Automation**

12.1. Save an arbitrary calibration configuration using the  button in the Membrane Potential Calibration Wizard.

12.2. In the Pipeline Parameters (Main Window Parameter Bar; the "Mitochondrial membrane potential measurement (TMRM/PMPI)" pipeline is activated) click the "Calibration configuration file name (*.ips)" parameter, and click the button appearing at the end of the line. Select the saved calibration file.

12.3. To automate saving the results give a filename in the "Output Excel Data save file name (*.xlsx)" using the string parser, such as =%LoadBaseName%%LoadStageNumber:2%.xlsx. See more about the string parser in the Main menu Help/"Help on Expression Evaluation". Optionally give a path, or set the default path to be used in the Main menu Files/"Set Folder Locations".

12.4. Use the pull down menu of the  button on the main toolbar or in the bottom of the Multi-Dimensional Open dialog to select "Run pipeline … on all stage positions" or "Run pipeline … on partial plate".

## DETERMINATION OF SPECTRAL CROSSBLEED BETWEEN TMRM AND PMPI

Spectral crossbleed can be calculated from a double-stained sample, given that the two spatially co-localized fluorophores behave differently in time. However, more precise determination of the crossbleed is possible with using single fluorophore stained samples and the same methods given below. Here the spectral crossbleed is calculated from the data set used for the potentiometric calibration.

1. Open only the baseline and the compete depolarization section of the experiment, by selecting the 01,10 and 11 files.
2. Select the "Prepare two channel time lapse for intensity analysis" pipeline in the Pipelines/"Intensity Measurements" main menu point.
3. The default parameters of the pipeline have been configured to work with this particular recording, otherwise set it up similarly to the pipeline used for image processing for the potentiometric calibration above.
4. Press the  button on the main toolbar or in the bottom of the Multi-Dimensional Open dialog to load and process the images.
5. Select the "Blind Spectral Unmixing with NMF" function from the Math menu.
6. Optionally set the "Write result to coefficient matrix" to Yes, and type a new matrix name (e.g. TMRM/PMPI).
7. Select both channels as Operand A (on the right of the function parameters) and press  in the toolbar. ***Note:*** *the order matters here, TMRM has to come first, then the PMPI. In the operands list Image windows appear in the order of their creation.*
8. Review the images that the spectral unmixing is correct, and no gross oversubtraction happen by the end of the experiment (appearing as negative values or black shadows around or within objects).
9. In the "Mitochondrial membrane potential measurement (TMRM/PMPI)" pipeline "Spectral Unmix Coefficient Matrix" give either the above entered new matrix name, or copy the coefficients from the "Spectral Unmix NMF Status" window. ***Note:*** *it is advised to run the calculation in multiple positions (the "Spectral Unmix NMF Status" also appears in the Excel Data Window) and calculate mean or median of coefficients.*
10. Enter coefficients into the "Spectral Unmix Coefficient Matrix" parameter of the pipeline. The matrix can be entered:
    a. As ={{1,"PMPI crossbleed into TMRM channel"},{"TMRM crossbleed into PMPI channel",1}} by writing the measured coefficients instead the quoted expressions.
    b. As a matrix name, where the named matrix is entered in the Main menu/Edit/Preferences/"Convolution kernels, Matrices" tab.