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1. Fluorescence Ratio Measurements

1.1 Introduction

In the following tutorial we will describe a whole-cell experiment with loading a ratiometric fluorescence dye (e.g. fura-2) through the patch pipette into the cell.

Before we start the detailed description how to set-up and configure PATCHMASTER, we will first outline the different phases of the experiment.

1. Approach of the cell with the patch pipette and formation of a seal.

2. During seal formation usually some fluorescence dye diffuses into the bath solution. Therefore, after seal formation we will detect a declining fluorescence signal since the dye molecules are diffusing in the bath solution away from the detection area. Due to dilution effects the fluorescence signal will decrease in the following tens of seconds. In this phase of the experiment, we have to wait for a stable fluorescence background, originating from auto fluorescence of the cell and dye in the tip of the patch pipette. (To reduce the contribution of fluorescence from dye molecules in the patch pipette you should restrict the field of fluorescence excitation and detection to the area of the cell.) When background fluorescence has been stabilized we will record this fluorescence as background fluorescence for later correction of the fluorescence ratio.

3. Establishment of the whole-cell configuration (break through) and recording of dye loading into the cell. During this phase of the experiment we start the fluorescence ratio measurement. We can follow the dye loading level and get first measurements of a fluorescence ratio.

For the experiment described above we recommend a minimal set of configurations. We will need:

- `Pulse Generator Sequences`: A sequence which applies a test pulse and records one pair of fluorescence values. We will name this sequence "TestFura". When executing this sequence data will not be stored. You will use this sequence e.g. during and short after seal formation for measuring the decline of the background fluorescence. Then we will use a sequence "RatioFura" which is very similar to the "TestFura" sequence but allows the storage of data and also calls a different Online Analysis method. This sequence will be used when recording a baseline, during resting times or e.g. during recovery periods.

- `Online Analysis`: We need two Online Analysis methods. The method "Background" will be used to analyze the fluorescence values and store them in a parameter value. The method "Ratio" will be used for the fluorescence ratio analysis during the experiment.

- `Protocols` **(optional)**: The `Protocol Editor` can be used to link the different `Pulse Generator Sequences` and switch from one phase of the experiment to the next. Depending on the design of the whole experiment one can use only one protocol containing all phases of the experiment. If more flexibility is needed one can create individual protocols or execute the `Pulse Generator Sequences` manually.

1.2 Configuring Patchmaster for Fluorescence Measurements

In the following chapter we describe all tasks which should be done before starting the final experiment. Our description outlines using photometry setup which accepts an analog voltage for wavelength control and provides an analog voltage (0 to 10V) proportional to the fluorescence signal. Usually, such system consists of a Monochrometer for fluorescence excitation and a Photomultiplier or Photo Diode as fluorescence detector. Such a

system can be controlled by PATCHMASTER using the `TILL Photometry` extension.

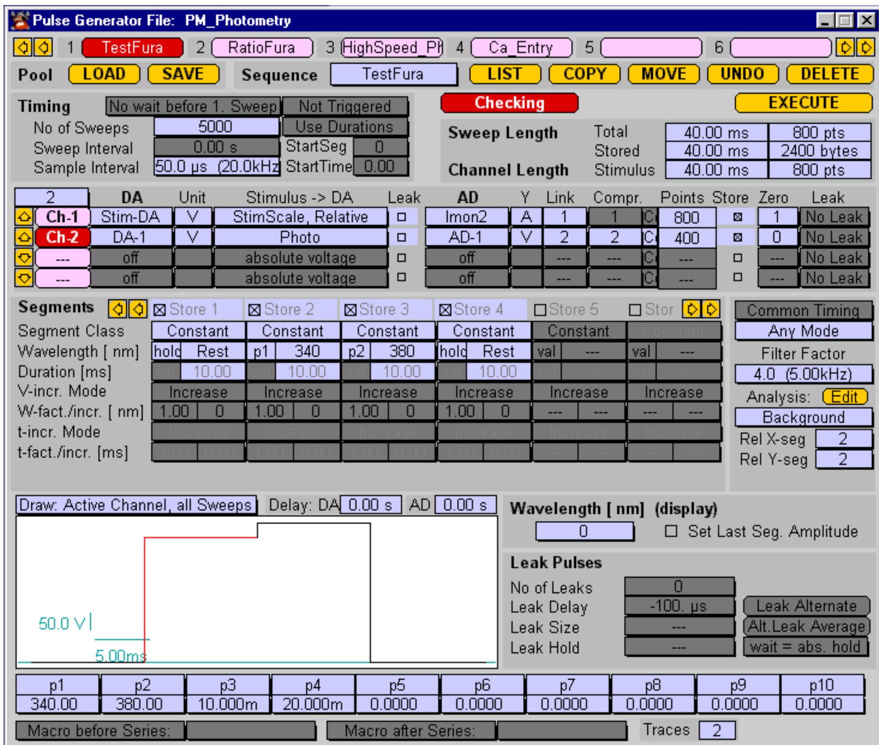
1.2.1 Turning on the `Photometry Extension`

After starting up the PATCHMASTER software open the `Configuration` window (`Windows` → `Configurations`) and select e.g. the `TILL` photometry extension (compare p. 109 Reference Manual) and follow the instructions given by the program. For calibration of the monochrometer please refer to the Reference Manual p. 114. Once the monochrometer is calibrated you can specify the excitation wavelength in PATCHMASTER directly. Conversion to the corresponding command voltage will be done by the photometry extension.

1.3 Editing the PulseGenerator Sequences

1.3.1 The Sequence "TestFura"

Open the `Pulse Generator` (`Windows` → `Pulse Generator`) and load the default protocol pool (`DefPgf_v9`). Delete all sequences except the sequence "TestSeries" and save the new Pulse Generator file under a new name, e.g. "Photometry". We will then rename the sequence "TestSeries" into "TestFura".



Please compare the settings of the first channel with the screenshot shown above. For stimulation you should use the "Stim-DA" and the `Stimulus` $\rightarrow$ `DA` should be set to "StimScale, Relative". As `AD` channel please select one of the current monitor (Imon) channels. The second channel we use for control of the fluorescence excitation device and record the fluorescence signal from the detector of the photometry system. As `DA` channel select the channel that was specified in the Photometry configurations as wavelength excitation channel ("Excit."). Here e.g. DA-1. As `Stimulus` $\rightarrow$ `DA` please select "use for Photometry". As `AD` channel please select the channel that has been specified as emission channel 1 ("E-mit 1") in the photometry configurations. Here AD-1. Optionally, one can use a data compression for the photometry channel.

For the first channel we use four segments, each with 10 ms duration and we apply a double pulse from the V-membrane relative +10 mV and then step to -10 mV and finally back to V-membrane.

Segments	☒ Store 1	☒ Store 2	☒ Store 3	☒ Store 4	☐ Store 5	☐ Stor
Segment Class	Constant	Constant	Constant	Constant	Constant	Constant
Voltage [mV]	hold V-mem	val 10	val -10	hold V-mem	val ---	val ---
Duration [ms]	val 10.00	val 10.00	val 10.00	val 10.00	val ---	val ---
V-incr. Mode	Increase	Increase	Increase	Increase	Increase	Increase
V-fact./incr. [mV]	1.00 0	1.00 0	1.00 0	1.00 0	--- ---	--- ---
t-incr. Mode	Increase	Increase	Increase	Increase	Increase	Increase
t-Fact./Incr. [ms]	1.00 0.00	1.00 0.00	1.00 0.00	1.00 0.00	--- ---	--- ---

For the second channel we use also four segments. Since we use this channel for Photometry, the amplitude of the stimulation can be entered in wavelength units, nm. We start from a resting wavelength of about 260 nm, then jump first to 340 nm and then to 380 nm and finally back to 260 nm.

Segments	☒ Store 1	☒ Store 2	☒ Store 3	☒ Store 4	☐ Store 5	☐ Stor
Segment Class	Constant	Constant	Constant	Constant	Constant	Constant
Wavelength [nm]	val 260	val 340	val 380	val 260	val ---	val ---
Duration [ms]	val 10.00	val 10.00	val 10.00	val 10.00	val ---	val ---
V-incr. Mode	Increase	Increase	Increase	Increase	Increase	Increase
W-fact./incr. [nm]	1.00 0	1.00 0	1.00 0	1.00 0	--- ---	--- ---
t-incr. Mode	Increase	Increase	Increase	Increase	Increase	Increase
t-Fact./Incr. [ms]	1.00 0.00	1.00 0.00	1.00 0.00	1.00 0.00	--- ---	--- ---

On the right side of the segments section we can specify additional parameters such as `Filter Factor`, `Analysis Method` and the `Relative X- and Y- segments`. We specify the `Analysis Method` "Background" which we will define later and set segment number 2 as `Relative segment`.

Common Timing	
Voltage Clamp	
Filter Factor	4.0 (5.00kHz)
Analysis: Edit	Background
Rel X-seg	2
Rel Y-seg	2

In the Timing section we enter a sample interval of e.g. 50.0 s and a large number of Sweeps (e.g. 5000).

Timing	No wait before 1. Sweep	Not Triggered	
No of Sweeps	5000	Use Durations	
Sweep Interval	0.00 s	StartSeg	Off
Sample Interval	50.0 µs (20.0kHz	StartTime	0.00

1.3.2 The Sequence "RatioFura"

The "RatioFura" sequence can be derived from the "TestFura" sequence. Please copy the "TestFura" sequence and name it "RatioFura". We will do two changes to this sequence:

- First, we enable "Store" of the sampled traces.

AD	Y	Link	Compr.		Points	Store	Zero	Leak
Imon2	A	1	1	C	800	☒	1	No Leak
AD-1	V	2	2	C	400	☒	---	No Leak
off		---	---	C	---	☐	---	No Leak
off		---	---	C	---	☐	---	No Leak

- Second, we change the Online Analysis Method from "Background" to "Ratio".

Common Timing
Voltage Clamp
Filter Factor
4.0 (5.00kHz)
Analysis: Edit
Ratio
Rel X-seg 2
Rel Y-seg 2

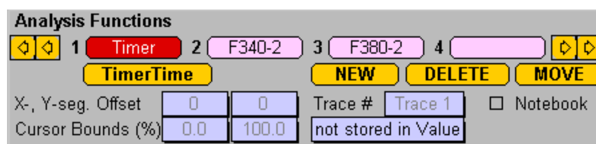
1.4 Editing the Online Analysis

In the upper part of the `Online Analysis` window, we create new `Analysis Methods`. We will start with the method "Background".

1.4.1 The "Background" Analysis Method

We select "Background" and configure three `Analysis Functions`. The first function we need is the TimerTime. We name this function just Timer. We will use this function to calculate the X-value for the online display.

For analysis of the fluorescence values we create two additional functions. We click on the `NEW` button in the `Analysis Function` section, select the function "Mean" from the Y-Analysis functions, enter a name for the function, e.g. F340, and click Done.



In the `Analysis Function` section we have to set the `Trace #` to "Trace 2", since the fluorescence signal is recorded with the second channel (see `Pulse Generator`). In addition, we will save the result in a `Value` that we can subtract the background fluorescence on a later time. Hence, we select "Store to Value-1". And we set the cursor bounds from 40 to 90%. The cursor bounds define the data range that is analyzed. The lower bound of the cursor bound has to be set to a value large enough to allow the fluorescence excitation source to settle at the specified wavelength. Note: The photometry extension of PATCHMASTER does not use the "dead time" anymore.

Now we create another "Mean" function with name "F380". In the `Analysis Function` section of the function "F380", we set the `Y-seg. Offset` to 1, since the F380 signal is measured in the next segment (see `Pulse Generator`). We again select "Trace 2" and store this result to "Value-2". We will also set the cursor bounds from 40 to 90%.

Table 1.1: The online functions of the background Analysis

Index	**Name**	**Description**
1	Timer	Time Time
2	F340-2	Mean of F340 Signal (relative segment of Trace 2), result will be stored in value-1 for later background correction
3	F380-2	Mean of F380 Signal (segment offset +1, trace 2), result will be stored in value-2 for later background correction

We display "F340-2" and "F380-2" versus "Timer" in separate graphs in the `Online Window 2`. We use "autoscaling after each sweep" for the y and x axis.

1.4.2 The "Ratio" Analysis Method

For the online analysis of the fluorescence ratio we need some additional functions. E.g. We need two constants representing the background fluorescence values (bg-F340, bg-F380). As `function type` select "Constant" from the Math group. Instead of "Use Constant Value" we select "Get Value-1". Then we have to subtract the background from the measured fluorescence and finally we calculate the ratio from the corrected fluorescence values.

Table 1.2: The online functions of the Ratio Analysis

Index	Name	Description
1	Timer	Time Time
2	F340-2	Mean of F340 Signal (relative segment of Trace 2)
3	F380-2	Mean of F380 Signal (segment offset +1, trace 2)
4	bg-F340	Background F340: Constant (Math), recall background value from value 1
5	bg-F380	Background F380: Constant (Math), recall background value from value 2
6	a2-a4	Subtract background (bg-F340) from F340-2
7	a3-a5	Subtract background (bg-F380) from F380-2
8	a6/a7	Ratio: divide result of function 6 by function 7

During the ratio acquisition we display the corrected fluorescence signals (a2-a4 and a3-a5) and the ratio (a6/a7) versus the timer time (Timer) in three separate graphs.

Here we use a fixed scaling for the y-axis (e.g. 0 - 2 V for the fluorescence signals and 0 - 1.5 for the ratio). You can also change the scaling during the experiment and adapt it to your needs. If you want to display the complete experimental run in the online window you can use "autoscaling after each sweep" for the x-axis scaling.

1.5 The Experiment with Photometry Measurements

Before you start the experiment you should open the `Online Window 2` and the `Protocol Editor` (To save some space you can reduce the window size of the `Protocol Editor` that only the first two lines are visible.).

First, approach the cell and form a giga-seal, as usual. Then, start the `Pulse Sequence` "TestFura" from the `Protocol Editor` window. You should see some fluorescence signal displayed in the `Online Window 2`.

The signal might be very close to zero since the cell is not loaded with dye yet. Wait until the fluorescence signal has stabilized. This remaining signal origin from auto-fluorescence of the cell and dye in the very tip of the patch pipette will be stored in the values 0 and 1 and will be subtracted from the fluorescence signals during a ratio measurement.

Now, stop the "TestFura" and establish the whole-cell configuration. Then start the "RatioFura" sequence for recording of the fluorescence signals. You will hopefully see a nice loading curve of fluorescence dye into the cell.

2. High-Speed Fluorescence Measurements

2.1 Introduction

For most application, the acquisition of one fluorescence data point per sweep is sufficient. However, some applications require recording fluorescence signals at higher time resolution simultaneously to the stimulus. In these case, e.g. repetitive fluorescence excitation stimuli must be applied in a single sweep and multiple fluorescence data points must be analyzed. In the following we demonstrate how to use the new data compression feature and virtual trace concept or PATCHMASTER to generate a new trace containing fluorescence ratios at high time resolution.

For a working example, we design an experiment in which we depolarize a cell for 300 ms to open e.g. Calcium channels. The calcium increase in the cell should be monitored simultaneously to the current recording.

2.2 Editing the pgf-File

In our working example (compare Fig. 2.1, the first channel (Ch-1) is used as "Stim-DA". We setup three segments with 100 ms, 300 ms and 50 ms duration each. The amplitude of the first and the third segment we set to holding. During the second segment we depolarize the cell to +20 mV. (The total sweep length is 450 ms. Since we have set the start time to 50 ms we actually store only 400 ms.)

> ***Note:*** *For this protocol we use a* `Sample Interval` *of 100 μs (10 kHz)*

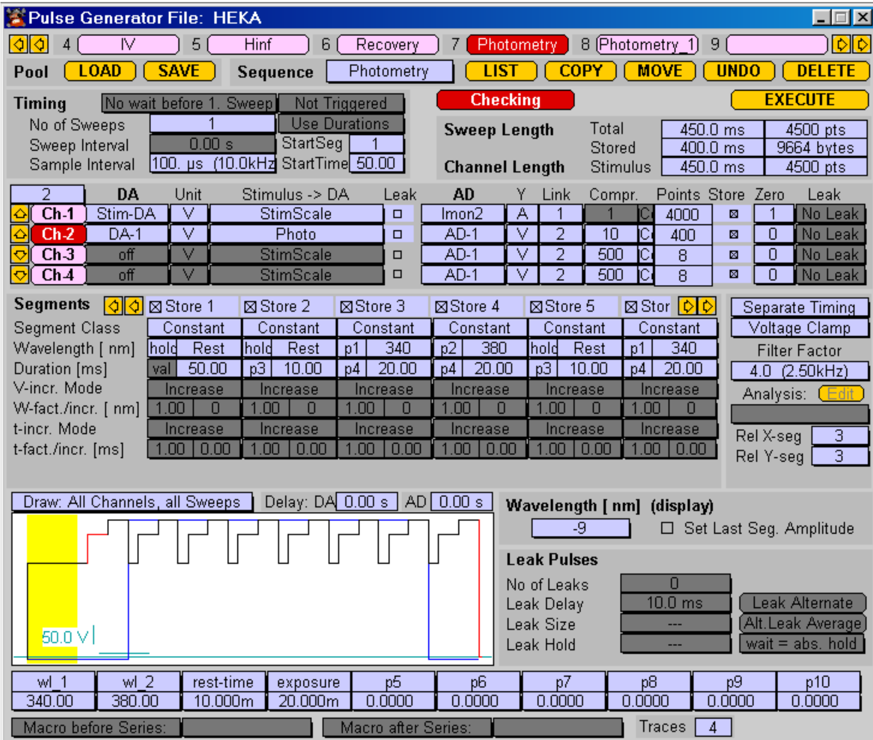


Figure 2.1: Pulse Sequence for high-speed ratio measurements

2.2.1 The Photometry Stimulation

In the second channel we define the photometry stimulus. Our aim is to apply several ratio stimulation cycles during the depolarization.

The photometry stimuli can be separated in three sections:

1. The start segment (segment 1). We set the fluorescence excitation to the resting wavelength to assure that our stimulation starts from a well defined wavelength.
2. The "parent" stimulation cycle (segments 2-4). A typical stimulation cycle for a ratiometric measurement consists of three segments:
   - a resting period
   - stimulation with wavelength one
   - stimulation with wavelength two

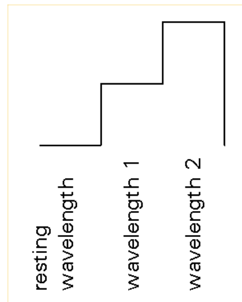


Figure 2.2: The fluorescence stimulus cycle

Therefore, we setup another three segments and make use of the pgf-parameters for parameterization of the parent stimulation cycle.

⊠ Store 2		⊠ Store 3		⊠ Store 4	
Constant		Constant		Constant	
hold	Rest	p1	340	p2	380
p3	10.00	p4	20.00	p4	20.00
Increase		Increase		Increase	
1.00	0	1.00	0	1.00	0
Increase		Increase		Increase	
1.00	0.00	1.00	0.00	1.00	0.00

- The resting wavelength can be parameterized using the "holding".
- For the two excitation wavelength we use the parameters 1 and 2. For better readability we rename those parameters to "wl_1" and "wl_2".
- We use parameter 3 to define the duration of the resting interval.
- The exposure times of the two excitation wavelength will be parameterized by parameter 4.

wl_1	wl_2	rest-time	exposure	p5	p6	p7	p8	p9	p10
340.00	380.00	10.000m	20.000m	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000

3. Finally we use the `Duplicate...` function to duplicate the three segments of the parent stimulation cycle. Open the context menu by click on the segment class of segment number 4. Select `Duplicate...` and enter "3" for `How many` and "8" for `How often`.

This pattern is defined as channel 2 and is output on DA-1.

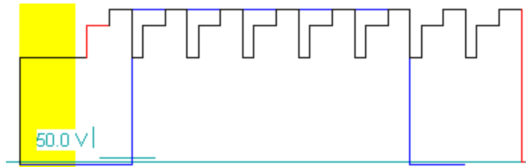


Figure 2.3: Stimulation protocol for voltage and fluorescence as shown in the pgf editor

We sample the fluorescence data from channel AD-1 and compress the data by a factor of 10 to yield one fluorescence point per ms. Trace 2 contains the fluorescence raw data with a time resolution of 1 ms. This trace can be displayed in the oscilloscope window together with e.g. the recorded current from the headstage.

However, this trace contains both the 340 nm and the 380 nm signals. This makes it very inconvenient for later ratio analysis.

Therefore, we will use an advanced compression feature to extract the 340 nm signals only.

2.2.2 Extraction of the F340 and F380 Signals

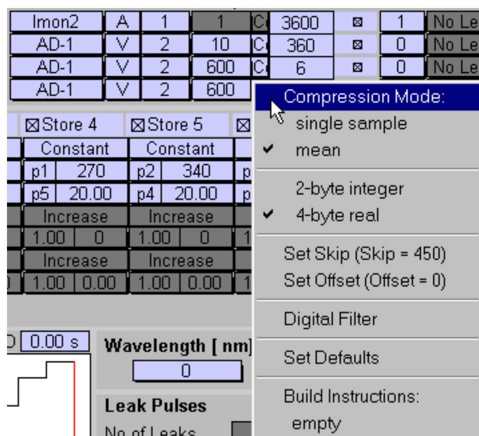
To calculate and store additional data traces we activate more channels (e.g. Channel 3 for F340 signal, channel 4 for F380 signal and channel 5 for the Ratio) by selection "AD-1" in the `AD` channel column of the channel settings. We link the channels 3 to 5 with channel 2.

In the following we describe how to configure the compression settings in order to separate the F340 and the F380 signal.

1. **Selection of an appropriate Compression Factor:** The compression factor has to be chosen that the compression interval (or range) covers a complete fluorescence stimulation cycle. (Number of recorded data points divided by the number of compressed data points gives the compression factor.) You should enter the compression factor in the field `Compr.` in the channel settings.

   In our example we are recording 4000 data points of channel 2 (AD-1, raw fluorescence), please compare figure 2.1. We would like to compress the whole sweep to yield 8 data points, one for each fluorescence stimulation cycle. The compression factor is calculates as follows: 4000 recorded data points / 8 compressed data points = 500.

2. **Compression Mode:** For selection of further compression Move the mouse tip over the "C" right to the `Compr.` field and open the context menu. You can select between "Single Sample" and "Mean". For the application described in this tutorial we advise to use the "Mean" compression mode.



3. **Set Skip:** With the `Set Skip` function you can skip a specific number of points of the compression interval before the compression starts.

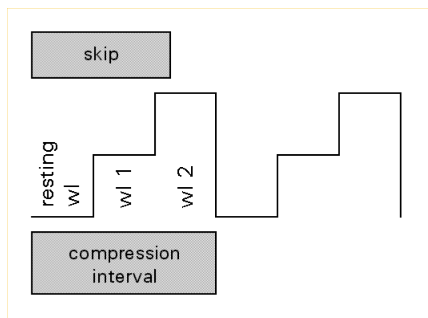


Figure 2.4: The compression range and skip range

For calculation of the `Set Skip` and `Set Offset` parameters the following rules are helpful:

- **Rule 1:** In a compression interval you can skip data points at the beginning of the interval only. (set Skip)
- **Rule 2:** Compression takes place in a range starting after the

skipped data points and ends at the end of the compression interval.

In case data points from the middle of the compression interval should be selected for compression (in our example the F340 signal), you must use the `Set Offset` function, see below.

In our example the Skip range should cover all points prior to compression including the so-called "dead time" of the fluorescence excitation source. The "dead time" is the time needed by the fluorescence excitation source to settle for a stable wavelength. The wavelength is set at the beginning of the segment. Usually it takes between 1 and 5 ms for settling on the destination wavelength.

In our example we have 10 ms resting wavelength, 20 ms exposure with 340 nm and 20 ms exposure with 380 nm. For the dead time we allow 5 ms.

In order to extract the F380 signal, we skip the resting wavelength, F340 stimulus and the first 5 ms of the F380 stimulus (dead time) and therefore set the `Set Skip` parameter to 350.

For extraction of the F340 signal we need in addition the `Set Offset` function, see below.

4. **Set Offset:** With the `Set Offset` function you can influence the start of the first compression interval. A negative offset moves the start of the compression interval to the left, a positive to the right.

   With other words, a negative offset adds dummy data points on the "negative time axis". Since compression starts at the first recorded point, the compression range is moved to the left (towards smaller times) with respect to the data points.

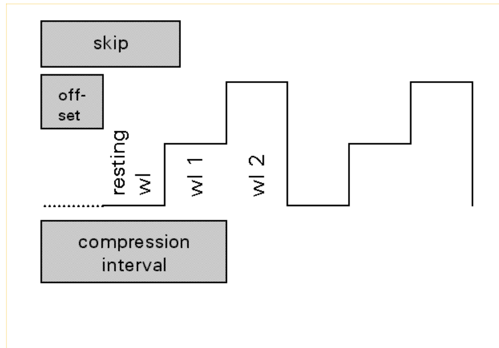
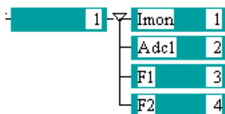


Figure 2.5: The offset range

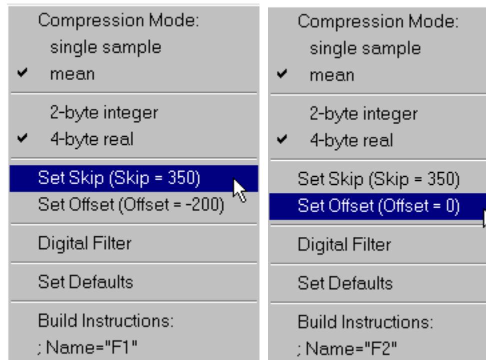
- **Rule 3:** The absolute value of the negative offset must be smaller or equal to the Skip value.

In order to extract the F340 signal we have to adjust the offset in such a way that the F340 signal is moved to the end of the compression interval (compare Rule 2). We therefore set the Offset to "-200". If we let the compression interval start 200 data points earlier (move the compression interval to the left by 200 points), the compression interval (duration 50 ms or 500 points) will end exactly after the F340 signal.

5. **Build Instruction:** You can use the build instruction to give the trace a name. Enter for example ";name="F2"". In the `Replay` window the names of the traces will be displayed e.g. as follows.



In summary the compression settings of channel 3 and 4 can look like:



Note: *The compression settings have to be adjusted if the sampling interval or a duration of one of the segments is changed.*

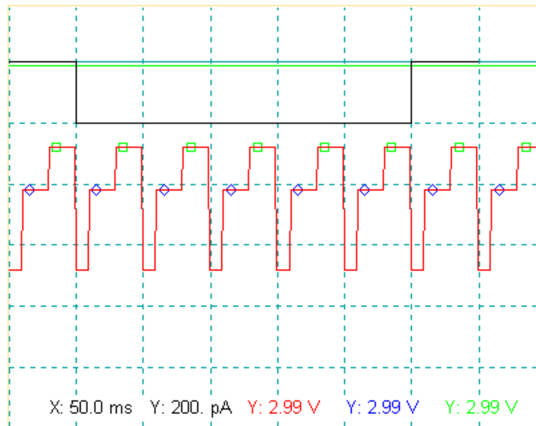
For a description how to easily (re-)calculate the compression settings please refer to the chapter 2.4 on page 20.

2.2.3 Calculation of the Ratio Trace

For calculation of the ratio trace we create a virtual trace in channel 5. (Set `AD` to "virtual". We choose the same compression factor as for channels 3 and 4. In the `Build Instructions` we enter "t3/t4;NAME="Ratio"". That means, trace 5 will be build by the ratio of data from trace 3 and trace 4 and the name of the new trace is "Ratio".

2.3 Display of the Data in the Oscilloscope window

After execution of the sequence the fluorescence data can be displayed in the `Oscilloscope` Window.



Which traces are displayed in the `Oscilloscope` window and which marker types are used can be selected from the menu `Display` → `Trace Properties`.

2.4 Calculation of Compression Settings

In the following we describe the formulas for calculation of the compression settings for a "high speed Fura-2 stimulation".

We assume that one stimulation cycle consists of three segments:

- the resting segment
- wavelength 1
- wavelength 2

We define the following parameters:

SamplingInterval	B5
RestingDuration (Segment 1)	B6
ExcitationDuration 1 (Segment 2)	B7
ExcitationDuration 2 (Segment 3)	B8
Beginning of Compression; DeadTime (cursor 1)	B9
End of Compression (cursor 2)	B10

Table 2.1: Timing Parameters of Fura-2 Stimulation; units [ms]

Once the above given parameters are specified, the compression settings can be calculated as follows:

CompressionInterval	=B6+B7+B8	[ms]
CompressionFactor	=B13/B5	[samples]
Wavelength 1		
Skip	=(B6+B7+B9+B10)/B5	[samples]
Offset	=-(B8+B10)/B5	[samples]
Wavelength 2		
Skip	=(B6+B7+B9+B10)/B5	[samples]
Offset	=-B10/B5	[samples]

Table 2.2: Results of Compression Calculator