# The *LockIn* extension of PULSE software

## 1. Introduction

Membrane capacitance ($C_m$) measurements have been used extensively to study exocytosis and endocytosis in individual cells. Currently, the most popular techniques for measuring changes in $C_m$ utilize a sinusoidal voltage stimulus and process the resulting sinusoidal current using a phase-sensitive detector or "lock-in amplifier" implemented either in hardware or software. A software-based phase sensitive detector is only a small part of the *LockIn* extension of PULSE software. *LockIn* together with an EPC9 comprises a unique "virtual" instrument with unprecedented capabilities for performing cellular admittance measurements without the need for external filters. Admittance can be measured even when the current monitor signal is profoundly altered with the use of capacitance and/or series resistance compensation. Thus estimates of the actual values of equivalent circuit parameters (rather than just relative changes) can be generated from *bona fide* admittance measurements.

### 1.1 "Sine+dc" (SDC) versus "piecewise-linear" (PL) techniques

Two different techniques are widely used for estimating changes in $C_m$ (see Gillis, 1995 for details) and are implemented as different "modes" by *LockIn*. Each takes a different approach towards resolving the dilemma that the use of a single sine wave stimulus provides only 2 pieces of information (magnitude and phase), whereas the equivalent circuit of a cell in the whole-cell recording configuration (figure 1) has 3 unknown parameters ($G_s$, $G_m$, and $C_m$).

In the first approach, which we call the "piecewise-linear" (PL) technique (Neher and Marty, 1982), no attempt is made to determine the actual value of the admittance or of any of the three equivalent circuit parameters. Instead, only *changes* in the parameters which result from measured changes in admittance are noted. The technique is based upon the approximation that small deviations in $C_m$ lead to linear changes in the admittance of the equivalent circuit which are orthogonal to changes in admittance introduced by small deviations in $G_m$ or $G_s$ (Neher and Marty, 1982; Joshi and Fernandez, 1988; Fidler and Fernandez, 1989). Thus if the output signal of the patch clamp amplifier is input to a phase-sensitive detector that is set to the appropriate phase angle, then the output of the instrument is linearly proportional to small changes in $C_m$, but relatively insensitive to changes in the resistive parameters. A signal is also generated from an orthogonal phase setting (the "G" signal), which reflects changes in either $G_s$ or $G_m$. In the PL mode of *LockIn*, "G" is split into two signals: $\Delta G_s$ and

$\Delta G_m$. Each of these signals are scaled appropriately to reflect changes in their respective parameters. **It is important to realize, however, that these two signals are actually differently scaled versions of the same trace. Changes in $G_s$ can not be distinguished from changes in $G_m$ in the PL mode.**

In the second approach, which we call the "sine + dc" method (SDC), the dc current, together with the real and imaginary parts of the admittance, provide the three necessary pieces of information (Lindau and Neher, 1988). Since the dc current is used, the reversal potential of the membrane conductance ($E_{rev}$) must be known. This limitation, however, is often not very serious since errors in the assumed value of $E_{rev}$ are not very critical under the common condition of $G_s >> G_m$ (Gillis, 1995).

Certainly the SDC method offers many advantages over the PL technique. The greatest advantage is that the SDC technique generates actual values of all three parameters rather than just relative changes. Why then is the PL technique still the most popular method for high-resolution recording of changes in $C_m$? One important reason is that making true admittance measurements requires one to keep track of many parameters (table 1). On the other hand, calibration of the "C" trace with the usual implementation of the P-L technique requires simply offsetting the capacitance compensation circuitry by a fixed amount. This procedure automatically takes into account the amplitude of the stimulus sinusoid, the gain of the patch-clamp amplifier, and other relevant factors. In addition, the required phase setting of the lock-in amplifier can be determined (albeit with potential errors; Gillis, 1995) by series resistance dithering ("phase tracking", Fidler and Fernandez, 1989) without a direct measurement of the phase shifts introduced by the patch-clamp amplifier. *Note: LockIn uses a calculated method for setting the phase and calibrating the traces in the PL mode that avoids the errors associated with series resistance dithering* (see section 2.11. and 3.1.).

One of the great advantages of PULSE software coupled to an EPC9 patch-clamp amplifier is that all of the instrumentation settings which can potentially affect an admittance measurement are "known" by the software. Thus the advantages of the SDC method can be realized with no additional record keeping burden imposed upon the user. **The sine + dc mode is the recommended or "standard" mode of operation of *LockIn*. The piecewise-linear mode was included to satisfy those users who may be more comfortable with this "traditional" type of measurement while making the transition to the superior SDC method.**

Whereas *LockIn* was designed with the EPC9 in mind, the software can also be used in a straightforward manner with any patch-clamp amplifier (see section 2.9.). Also, the discussion throughout the manual emphasizes acquisition of $C_m$ data, however, estimates of the other two equivalent circuit parameters ($G_s$ and $G_m$) accompany each $C_m$ estimate when the SDC mode is used.

## 1.2 High versus low time resolution measurement of $C_m$

Different experimental conditions typically lead to different rates of $C_m$ changes. For example, increases in $C_m$ occur over many seconds to minutes when buffered $Ca^{2+}$ solutions or other stimulatory substances are introduced into the cell through the patch pipette. Decreases in $C_m$ reflecting endocytosis also often occur on a relatively slow time scale. On the other hand, high time resolution estimation of $C_m$ is desirable to follow exocytosis immediately before and after a depolarizing pulse and is essential to record exocytosis evoked by flash photolysis of caged $Ca^{2+}$. Matching the acquisition rate to the expected rate of change in $C_m$ can dramatically reduce storage requirements. In addition, the architecture of PULSE software places constraints on how long high speed acquisition of $C_m$ can occur without "gaps". This situation has led to two distinct acquisition modes within *LockIn*.

High speed acquisition produces a $C_m$ estimate for every sine wave cycle, i.e. typically 1000 points per second. This high rate of acquisition can be supported without interruption for the duration of a sweep -- typically 1 - 2 seconds, (the maximum sweep duration corresponds to 16k samples) and can be displayed in the *Oscilloscope* window. The raw data of each sweep ('Imon') must be stored in order to save the $C_m$ values at high time resolution (high time resolution $C_m$ values are actually *calculated* from the raw data, not stored). Gaps in acquisition occur between sweeps which can be longer than one second if data are written to hard disk. (See section 4.2. for tips on minimizing gaps).

Low time resolution acquisition is best performed using the XChart extension of PULSE software. **The XChart extension is highly recommended for $C_m$ measurements using *LockIn*.** Here, data from an entire sweep are averaged to result in one $C_m$ point per sweep. The $C_m$ values (as well as the $G_s$ and $G_m$ values, if desired) are displayed and stored by the XChart extension, therefore the raw data sweeps do not have to be saved -- resulting in a tremendous reduction in storage requirements. Typically, 100 sine wave cycles are output with each sweep and a gap of about 100 ms occurs between sweeps, resulting in an acquisition rate of about 5 Hz. If you don't have XChart, you can send the values to the notebook window which can be later saved to disk. Low time resolution $C_m$ values can be displayed in the on-line analysis window, but this option is of limited practicality. On-line analysis values are not stored, but calculated from the raw traces. Therefore, the raw current traces have to be saved in order to use this display option for replay of data.

The two acquisition rates can be easily mixed during the course of an experiment. Consider the example of depolarization-evoked increases in $C_m$. Sweeps which contain depolarizing pulses (and therefore contain voltage-gated currents such as $I_{Ca}$) can be saved to preserve high time resolution $C_m$ data immediately before and after each pulse. These saved sweeps can be linked to series which are not saved, but which produce sweeps of sine waves for low time resolution $C_m$ acquisition during the periods between depolarizing pulses. See sections 2.1. - 2.7. for detailed examples.

Note that there is no explicit "toggle" switch between slow and fast modes; the two types of acquisition proceed concurrently. "Fast" acquisition occurs automatically for sweeps that are stored. In addition, $C_m$ values are *always* averaged on a sweep-by-sweep basis with the result sent to XChart and/or the notebook if these options are selected. *XChart only receives one* $C_m$ *point per sweep* (the averaged value). Thus if XChart is set to record $C_m$ data, then each sweep that is saved in PULSE is recorded both in high time resolution (and displayed in the *Oscilloscope* window, if desired) and is represented by a single $C_m$ point in XChart. Both XChart data and high time resolution data can be exported for analysis in Igor. In addition, the high time resolution data can be inserted into the low time resolution traces within Igor by using a macro ("Combine") that is available without charge from HEKA. It should be pointed out however, that we have rarely found the need to do this because all of our quantitative analysis of depolarization-evoked exocytosis is performed within Igor using only high time resolution sweeps (see section 2.10.). ("Depol", our analysis macro, is also available without charge from HEKA). We use XChart for looking at endocytosis, recording other traces of interest (e.g. FURA-2 fluorescence signals) and for providing a hard copy output summarizing an experiment for inclusion in a lab notebook.

# 2. Tutorial

The following section gives specific examples in order to provide a "feel" of how *LockIn* operates using a model circuit. **Point-by-point explanation of the various menus and windows which control the operation of *LockIn* is reserved for section 3.**

## 2.1. Basic measurements with and without CSlow compensation

Here we make our first capacitance measurement using a model circuit and look at how *LockIn* works with together with capacitance compensation.

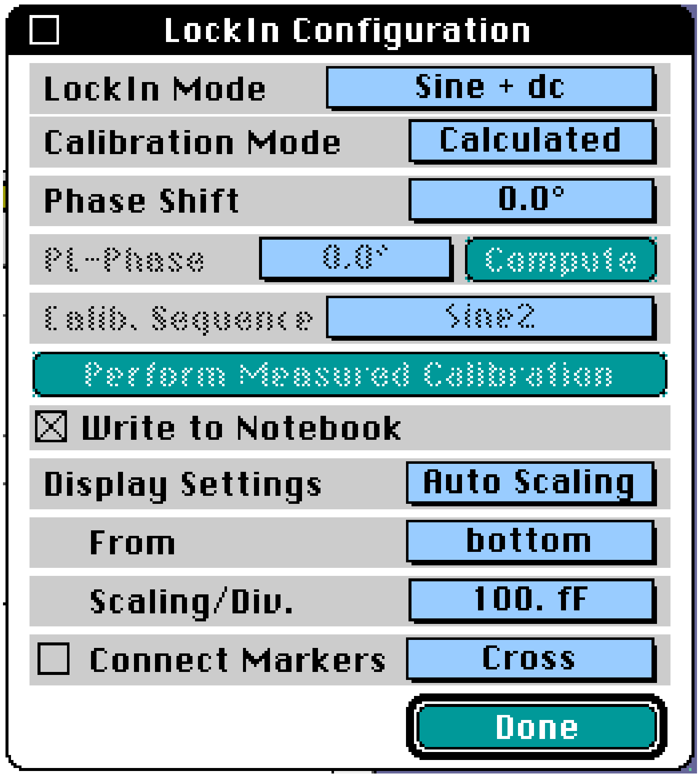
1) Activate the *LockIn* extension of PULSE

This step only has to be done the first time one uses *LockIn*.

Launch PULSE and select *Activate LockIn* from the *Pulse* pull down menu. The software instructs you to store the Configuration File, exit PULSE, and then restart PULSE. Select *OK*, then *Quit*. You will be prompted to select a Configuration File. The configuration file that is read upon launch of PULSE is named *DefaultPulse.set* and must be located in the *Pulse* folder. After selecting this, select *Save*. After agreeing to *Replace* the existing *DefaultPulse.set*, launch PULSE again. You should now see the command *LockIn Configuration* under the *Pulse* pull down menu.

2) Set the *LockIn Configuration*

Select *LockIn Configuration* from the *Pulse* pull down menu (or press <Command><->). Set the parameters to the following values:



LockIn Configuration

LockIn Mode: Sine + dc

Calibration Mode: Calculated

Phase Shift: 0.0°

PL-Phase: 0.0°

Calib. Sequence: Sine2

☒ Write to Notebook

Display Settings: Auto Scaling

From: bottom

Scaling/Div.: 100. fF

☐ Connect Markers: Cross

3) Attach the MC-9 model circuit to the head stage of the EPC9.

Set the switch to to the 10 MΩ setting ("pipette").

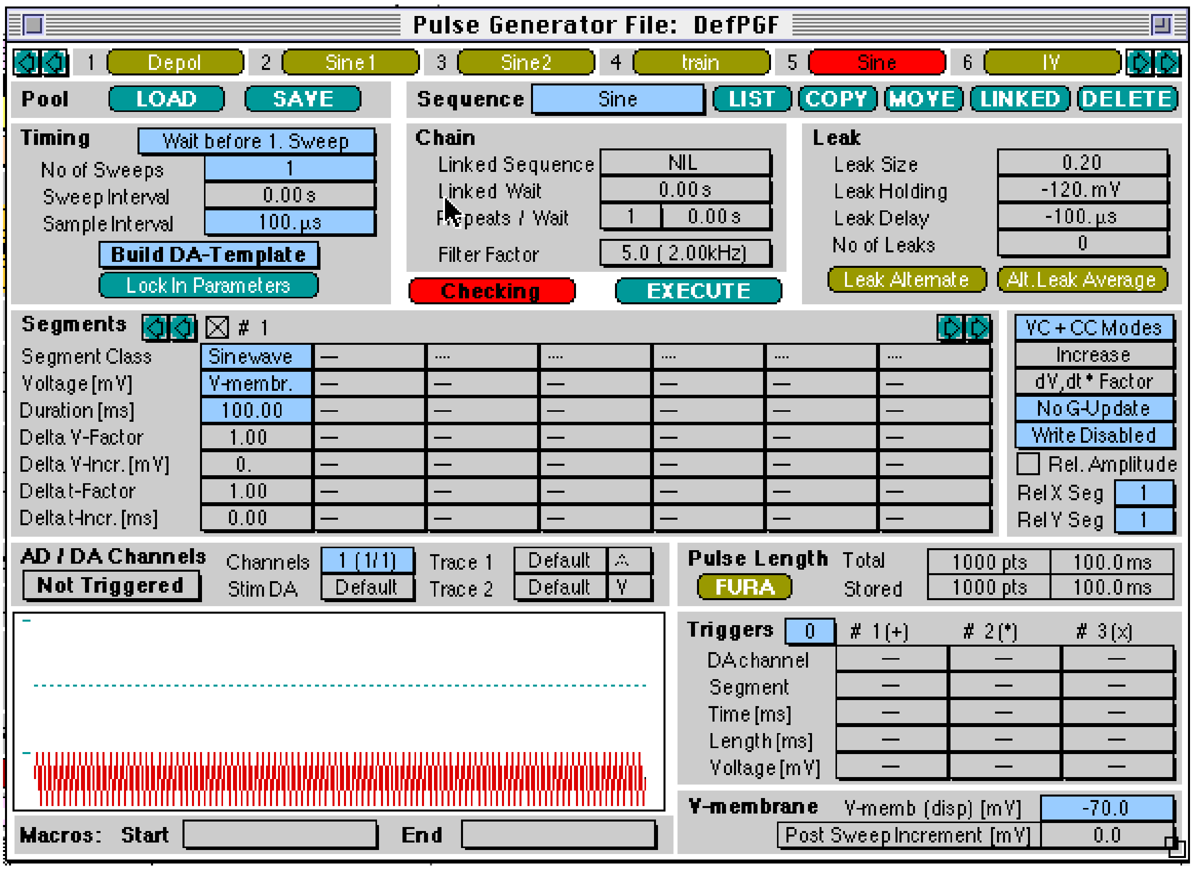
4) Set the *EPC9 Patch-Clamp* window.

a) Perform an *Auto Voffset* compensation (press 'Z').
b) Set the switch on the MC-9 model circuit to the middle position ("on cell").
c) Perform an *Auto CFast* compensation (press 'A').
d) Set the switch on the MC-9 to the 0.5 GΩ setting ("whole cell").
e) Set the *Gain* to *1.0 mV/pA*, *V-membrane* to *-50 mV*. Select *Imon2* and *Whole Cell* modes. Set *Filter 1* to *Bessel 10 kHz*, *Filter 2* to *Bessel 2.0 kHz*, and *Stim* to *20 µs*. Leave *CSlow*, *RsComp*, *Leak Comp* and *Stim, External* in the *Off* position.

5) Make sure the *Notebook* window is open. Select *Buffered Output* from the *Notebook* pull down menu.

6) Create a simple sine wave series in a PGF (Pulse Generator File).

Open the *Pulse Generator* window from the *Pulse* pull down menu (or press F12). Duplicate the following settings to create a series named "Sine":



Sequence: Sine

**Timing**: Wait before 1. Sweep
No of Sweeps: 1
Sweep Interval: 0.00 s
Sample Interval: 100. µs

**Chain**
Linked Sequence: NIL
Linked Wait: 0.00 s
Repeats / Wait: 1 / 0.00 s
Filter Factor: 5.0 ( 2.00kHz)

**Leak**
Leak Size: 0.20
Leak Holding: -120. mV
Leak Delay: -100. µs
No of Leaks: 0

**Segments** ☒ # 1

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| Segment Class | Sinewave | — | .... | .... | .... | .... | .... |
| Voltage [mV] | V-membr. | — | — | — | — | — | — |
| Duration [ms] | 100.00 | — | — | — | — | — | — |
| Delta V-Factor | 1.00 | — | — | — | — | — | — |
| Delta V-Incr. [mV] | 0. | — | — | — | — | — | — |
| Delta t-Factor | 1.00 | — | — | — | — | — | — |
| Delta t-Incr. [ms] | 0.00 | — | — | — | — | — | — |

☐ Rel. Amplitude
Rel X Seg: 1
Rel Y Seg: 1

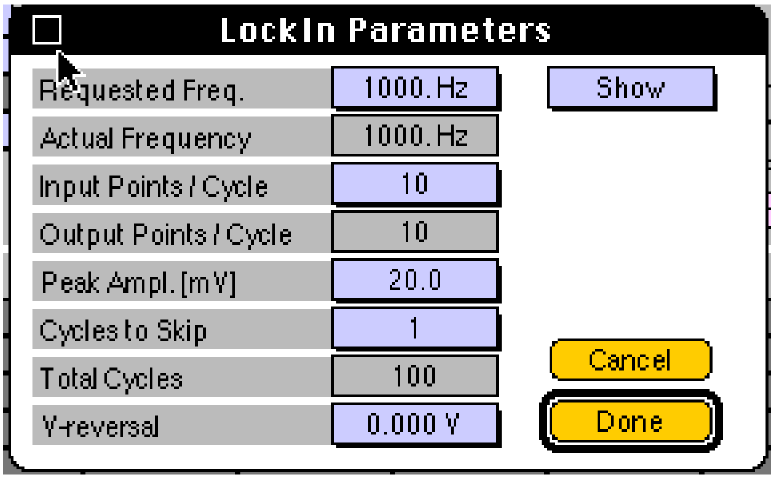
**AD / DA Channels**
Channels: 1 (1/1)
Stim DA: Default
Trace 1: Default A
Trace 2: Default V

**Pulse Length**
Total: 1000 pts, 100.0 ms
Stored: 1000 pts, 100.0 ms

| Triggers 0 | # 1(+) | # 2(*) | # 3(x) |
|---|---|---|---|
| DA channel | — | — | — |
| Segment | — | — | — |
| Time [ms] | — | — | — |
| Length [ms] | — | — | — |
| Voltage [mV] | — | — | — |

**V-membrane**
V-memb (disp) [mV]: -70.0
Post Sweep Increment [mV]: 0.0

Note that *Write Disabled* is selected. Pressing the *Lock In Parameters* button opens up a window, duplicate the following settings:



| | |
|---|---|
| Requested Freq. | 1000. Hz |
| Actual Frequency | 1000. Hz |
| Input Points / Cycle | 10 |
| Output Points / Cycle | 10 |
| Peak Ampl. [mV] | 20.0 |
| Cycles to Skip | 1 |
| Total Cycles | 100 |
| V-reversal | 0.000 V |

7) Execute the new "Sine" series by pressing the corresponding button in the *Oscilloscope* window. The series outputs 100 cycles of a 1 kHz sine wave voltage, measures the resulting sinusoidal current, and outputs averaged estimates to the *Notebook* window. Typical values are:

A: 67.26nS B: 90.28nS b: 1.982nS Phase: 53.3°
RS: 5.205MΩ RM: 499.3MΩ CM: 22.340pF

'A' and 'B' represent the Real and Imaginary components of the admittance, respectively, which include corrections for the attenuation and phase shift introduced by the recording instrumentation (see section 3.1.). 'b' is the dc conductance.

Note that these values were obtained with capacitance compensation *off*. Often, it is desirable to nullify the capacity current transients that accompany depolarizing pulses in order to prevent the amplifier from saturating.

8) Perform an *Auto CSlow* compensation (press 'B').

Execute the "Sine" series again. The values output to the *Notebook* window are now slightly different. Typical values are:

RS: 5.53 MΩ RM: 504 MΩ CM: 21.69 pF

**Why are the values different?** Note that the sinusoidal current displayed in the *Oscilloscope* window is very different depending on if CSlow compensation is *on* or *off*. Use of CSlow compensation "nulls out" the bulk of the sinusoidal current just as it eliminates capacitive current transients elicited by voltage steps. However, the original "unnulled" current is needed to estimate the three equivalent circuit values. Therefore, the software "adds back" the nulled currents when processing the estimates (see Gillis, 1995 for details). *LockIn* can calculate what to add back because it always knows the *C-Slow* and *R-Series* instrument settings when an EPC9 is used. This process is generally quite accurate, however, it is not perfect. CSlow compensation and the model circuit may not behave ideally (neither, of course, do real cells), and small phase errors within *LockIn* can occur. Estimates generated by *LockIn* with CSlow compensation on can be thought of as a "hybrid" of estimates made by nulling current transients with CSlow circuitry and of estimates made with sine waves. The estimate from CSlow circuitry dominates the *LockIn* estimate when the bulk of the sinusoidal currents are nulled. (Note that *LockIn* estimates generated above are essentially identical to the *C-Slow* and *R-Series* settings.) This situation has an important consequence:

**An *Auto CSlow* update can result in small jumps in $C_m$ estimates generated by *LockIn* that have nothing to do with exocytosis or endocytosis.** Once you are aware of this possibility, it seldom creates an actual problem. *Auto CSlow* updates should be performed during "waiting" periods between depolarizing pulses, **not immediately before, after or during pulses** (for example) and can be avoided entirely if $C_m$ changes are evoked by stimulatory substances introduced into the cell through the patch pipette. This is only an issue for low time resolution (XChart) acquisition. **High time resolution measurements of changes in $C_m$ are not affected by CSlow updates immediately before or after the sweep**.

9) Set CSlow = 17.0 pF, RSeries = 7 MΩ, Range = 1000 pF

Execute the "Sine" series again. Typical values sent to the *Notebook* window are:

RS: 5.46 MΩ RM: 503 MΩ CM: 21.82 pF

Note that even though both compensated values are in error by more than 20%, the values calculated by *LockIn* are within 1.5% of the values obtained with "correct" compensation.

## 2.2. High time resolution display

Here we look at how we can display the results we obtained in the previous section with high time resolution.

1) From the *Display* pull down menu, select *Display mode: I vs. t + LockIn*. Also select *Labeling: Grid + Labels*.

2) Open the *PGF* window (F12) and change the "Sine" series from *Write Disabled* to *Write Enabled*.

3) Make sure the *Store* button in the *Oscilloscope* window is selected.

4) Execute the "Sine" series. You will see a red trace with one $C_m$ value for every sine wave cycle. (At the present time, high time resolution $G_m$ and $G_s$ traces are not available for display in the *Oscilloscope* window, however, they can be exported to Igor for "off line" display.)

5) The bottom half of the *LockIn Configuration* window (<Command><->) controls the display of the $C_m$ trace in the *Oscilloscope* window. Try varying the parameters, then select the stored trace from the *Replay* window to update the *Oscilloscope* window.

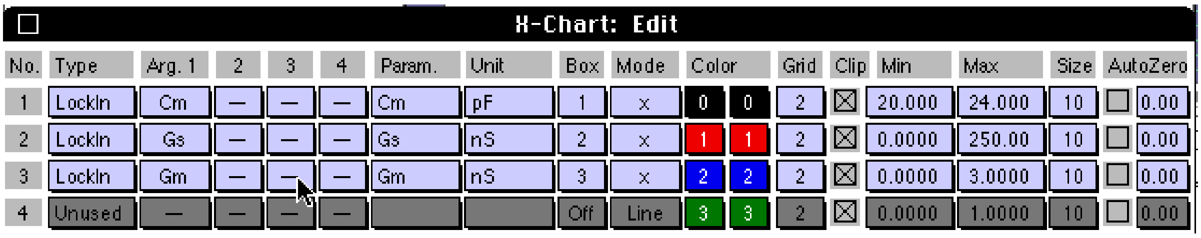
## 2.3. Sending values to XChart

The following example shows how to run the software for low time resolution "continuous" recording of $C_m$, $G_s$ and $G_m$. This type of acquisition would be appropriate for measuring changes in $C_m$ elicited by substances (e.g. GTP-g-S, buffered $Ca^{2+}$) introduced into the cell through the patch pipette.

1) If you haven't done so already, *Turn X-Chart On* in the *Pulse* pull down menu.

2) From the *X-Chart* pull down menu, select *Edit Traces and Param.* (or press <Command><4>).

3) Copy the following into the first 3 lines of the *X-Chart: Edit* window:



X-Chart: Edit

| No. | Type | Arg. 1 | 2 | 3 | 4 | Param. | Unit | Box | Mode | Color | | Grid | Clip | Min | Max | Size | AutoZero | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | LockIn | Cm | — | — | — | Cm | pF | 1 | x | 0 | 0 | 2 | ☒ | 20.000 | 24.000 | 10 | ☐ | 0.00 |
| 2 | LockIn | Gs | — | — | — | Gs | nS | 2 | x | 1 | 1 | 2 | ☒ | 0.0000 | 250.00 | 10 | ☐ | 0.00 |
| 3 | LockIn | Gm | — | — | — | Gm | nS | 3 | x | 2 | 2 | 2 | ☒ | 0.0000 | 3.0000 | 10 | ☐ | 0.00 |
| 4 | Unused | — | — | — | — | | | Off | Line | 3 | 3 | 2 | ☒ | 0.0000 | 1.0000 | 10 | ☐ | 0.00 |

4) From the *X-Chart* pull down menu, make sure *Active: After each sweep* is checked. The other options under *Active* should **not** be checked. *LockIn* parameter estimates are only updated once per sweep, so there is no point in having values sent to XChart in-between sweeps.

5) Select *Graph* from the *X-Chart* pull down menu (or press <Command><2>) and size and place the window where desired.

6) Select *Data and Comments* from the *X-Chart* pull down menu (or press <Comand><5>) and size and place the window where desired.

7) The "Sine" series should be set to *Write Disabled*.

8) In the *X-Chart: Control* window, press the *Reset*, then the *Run* button.

9) In the *EPC9 Patch-Clamp* window, under *Test Pulse* type, select *TestSeries*. For the Name of the Test Pulse Series, type "Sine". This will cause the series "Sine" to be repeatedly executed. Each sweep produces one "X" (the averaged value) for each of the equivalent circuit parameters in the *X-Chart: Graph* window. The numerical values are also sent to *X-Chart: Data and Comments*. Note that execution of the sweeps stops if the *EPC9 Patch-Clamp* window is not active.

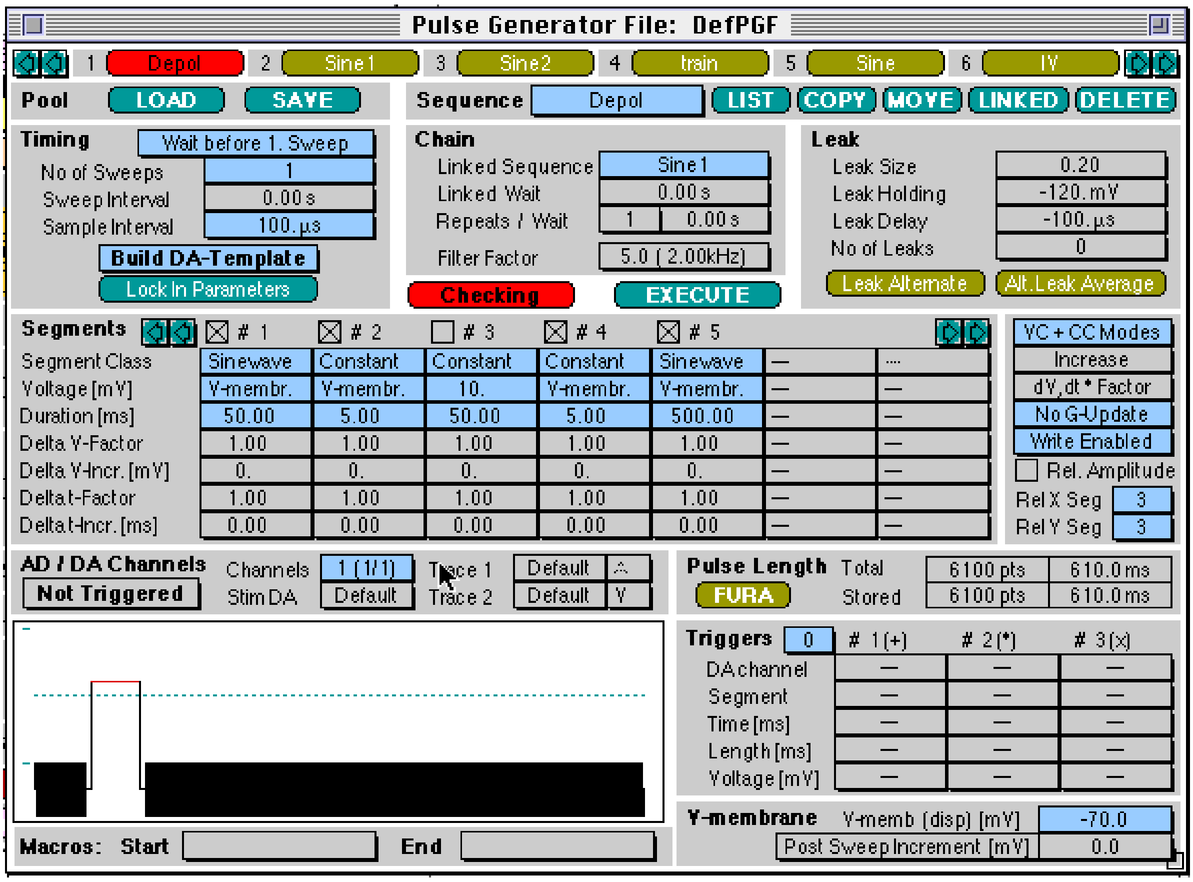
10) The "Sine" Test Series can run faster if the high time resolution $C_m$ display is disabled. Section 2.2. showed how the oscilloscope display is enabled or disabled globally from the *Display* pull down menu. However, the oscilloscope display can also be disabled or enabled for individual series within the *PGF: LockIn Parameters* window. From the *PGF* window (F12) push the *LockIn Parameters* button within the "Sine" series to activate the *LockIn Parameters* window. Toggle *Show* to *No Show*.

11) You can also speed up the *Test Series* by disabling the writing of *LockIn* parameters to the *Notebook* window. Open the *LockIn Configuration* from the *Pulse* pull down menu (or press <Command><->) window and remove the check from the *Write to Notebook* check box. **Given that low time resolution data are stored by XChart, the *Write to Notebook* option is usually only enabled during testing**.

## 2.4. A simple example of recording depolarization-evoked increases in $C_m$.

Here we show how low and high time resolution recording can be mixed.

1) Copy the "Sine" series in the PGF window (F12) to create a new series named "Depol". Press the *Move* button and reassign "Depol" to position "1". Configure "Depol" as follows:



Sequence: Depol

**Timing**: Wait before 1. Sweep
No of Sweeps: 1
Sweep Interval: 0.00 s
Sample Interval: 100. µs

**Chain**
Linked Sequence: Sine1
Linked Wait: 0.00 s
Repeats / Wait: 1 | 0.00 s
Filter Factor: 5.0 ( 2.00kHz)

**Leak**
Leak Size: 0.20
Leak Holding: -120. mV
Leak Delay: -100. µs
No of Leaks: 0

| Segments | ☒ # 1 | ☒ # 2 | ☐ # 3 | ☒ # 4 | ☒ # 5 | | |
|---|---|---|---|---|---|---|---|
| Segment Class | Sinewave | Constant | Constant | Constant | Sinewave | — | .... |
| Voltage [mV] | V-membr. | V-membr. | 10. | V-membr. | V-membr. | — | — |
| Duration [ms] | 50.00 | 5.00 | 50.00 | 5.00 | 500.00 | — | — |
| Delta V-Factor | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 | — | — |
| Delta V-Incr. [mV] | 0. | 0. | 0. | 0. | 0. | — | — |
| Delta t-Factor | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 | — | — |
| Delta t-Incr. [ms] | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | — | — |

☐ Rel. Amplitude
Rel X Seg: 3
Rel Y Seg: 3

**AD / DA Channels**: Not Triggered
Channels: 1 (1/1)
Stim DA: Default
Trace 1: Default ^
Trace 2: Default V

**Pulse Length**
Total: 6100 pts | 610.0 ms
Stored: 6100 pts | 610.0 ms

| Triggers 0 | # 1(+) | # 2(*) | # 3(x) |
|---|---|---|---|
| DA channel | — | — | — |
| Segment | — | — | — |
| Time [ms] | — | — | — |
| Length [ms] | — | — | — |
| Voltage [mV] | — | — | — |

**V-membrane**
V-memb (disp) [mV]: -70.0
Post Sweep Increment [mV]: 0.0

Note that *Write Enabled* is selected. Also, the *Rel*(evant) *Y Seg* is "3" -- the segment with the depolarizing pulse. This will later signal an analysis program that the amplitude and duration of this segment are to be noted. The *LockIn Parameters* of "Depol" should be identical to "Sine" with one important difference: "Sine" has *No Show* selected whereas "Depol" should have *Show* enabled.

2) Make sure that *Display Mode* in the *Display* pull down menu is set to *I vs. t + LockIn*.

3) The *Store* button in the *Oscilloscope* window should be selected.

4) Activate the *Configuration* window (*Configuration* in the *Pulse* pull down menu or press F11). *Wait after Stim.* should **not** be checked.

5) Perform an *Auto CSlow* compensation (press 'B').

6) In the *X-Chart: Control* window, press the *Reset,* then the *Run* button.

7) In the *EPC9 Patch-Clamp* window, under *Test Pulse* type, select the "Sine" series. The "Sine" series is now repeatedly executed. Each execution sends one set of *LockIn* estimates to XChart.

8) At any time, trigger the execution of the "Depol" series. The best way to do this is to simply press the number that corresponds to "Depol" (in this case "1"). If the series is launched from the keyboard while the *EPC9 Patch-Clamp* window is active, then the *TestSeries* ("Sine") resumes repeated execution as soon as "Depol" finishes.

Thus "Sine" produces low time resolution points that are sent to XChart. The raw data sweeps are not saved ("Sine" is set to *Write Disabled*), nor are the $C_m$ values of "Sine" displayed in the *Oscilloscope* window (*No Show* is selected in the *LockIn Parameters* window of "Sine"). When "Depol" is executed, high time resolution $C_m$ values are displayed in the Oscilloscope window (*Show* **is** selected in the *LockIn Parameters* window of "Depol"). for an "on-line" look at the time course of $C_m$ changes in the 500 ms that follows the depolarization. Low time resolution acquisition resumes as soon as "Depol" data are written to disk. Note that the $C_m$ trace in the *Oscilloscope* window is blanked during the constant segments, including the depolarizing pulse. Naturally, $C_m$ can only be estimated during *Sinewave* segments. (Depolarizing pulses can be selected with superimposed sine waves, but this is not recommended since nonlinear conductances will invalidate $C_m$ estimates.) The raw data traces from "Depol" sweeps are stored so that the voltage dependent currents are saved as well as the high time resolution *LockIn* parameters (which are actually calculated from raw data-- not stored).

## 2.5. Depolarizing pulses given at regular intervals

With the type of acquisition illustrated in section 2.4., depolarizing pulses are triggered at will by a user keystroke. It is often desirable, however, to have the pulses triggered automatically at regular intervals (e.g. once per minute) without relying on a manual input. One way to do this is illustrated in the following example.

1) *Copy* the "Sine" series in the PGF window (F12) to create a new series named "Sine1". *Move* "Sine1" to position "2". Change the *No of Sweeps,* from "1" to "50". The *Sweep Interval* should be 0.0 s. The other settings remain the same (*Write Disabled* should be selected and *No Show* should be selected under the *LockIn Parameters* settings).

2) Copy the "Sine1" to create a new series named "Sine2". *Move* "Sine2" to position "3". Specify "Depol" as the *Linked Sequence.* Change *No G-Update* to *Update C-Slow before Series.*

3) Select "Depol" and specify "Sine1" as the *Linked Sequence.*

4) Select "Sine1" and specify "Sine2" as the *Linked Sequence*.

5) In the *X-Chart: Control* window, press the *Reset,* then the *Run* button.

6) Start the series "Sine1" by pressing "2" from the keyboard. "Sine1" will execute 50 times with a minimal gap between sweeps because the (intentional) *Sweep Interval* is "0.0s". The gap will vary with the computer that is used; it is about 110 ms with a Mac Quadra 800. Each sweep will send a single $C_m$ point to XChart. After "Sine1" finishes, control passes to "Sine2", which executes an *Auto CSlow* update before its 50 sweeps are executed. Finally, "Depol" is executed and the loop begins again with the link to "Sine1". **Since the loop is infinite, the only way to terminate execution is with the *Break* or *Stop* buttons in the *Oscilloscope* window.**

The reason that two different "Sine" series are used between "Depol"s is that this allows a CSlow update in the middle of the "resting period" between depolarizations. As noted in Example 1, small jumps in reported $C_m$ values can accompany CSlow updates, so it is a good idea to have the updates well separated in time from depolarizations. We repeat again: **This is only an issue for low time resolution (XChart) acquisition**. High time resolution measurements of changes in $C_m$ are not affected by CSlow updates immediately before or after the sweep. CSlow updates are not performed to aid $C_m$ measurements, but rather to null capacity current transients that accompany the depolarizing pulses.

The choice of 100 sine wave sweeps between depolarizations in this example is arbitrary. You will want to empirically determine for your individual computer the number of sweeps that are required to produce the desired "resting period" between pulses.

## 2.6. Generating C-I-V curves or $\Delta C_m$ versus duration curves.

An important consideration for designing protocols for looking at the relationship between changes in $C_m$ and pulse potential or duration is that **the pulse generator presently does not allow sine wave sweeps to occur during *Sweep Interval*s or other waiting intervals.** Therefore, if a C-I-V curve (for example) is generated from a single series using a *Delta V-Incr*(ement), then no *LockIn* information is sent to XChart between pulses to indicate what is happening to $C_m$ (or $G_s$, $G_m$) between sweeps. Of course the same problem would occur if you use a *Delta t-Incr.* to try the efficacies of different pulse durations in eliciting $\Delta C_m$s. These types of experiments can be performed in two different ways:

i) If you aren't worried about what occurs between pulses, then use the single series with multiple pulses separated in time by a sufficiently long *Sweep Interval.* In this case, there is no need to run XChart. Of course, you will want to put a short *Sinewave* segment before the pulse and a long *Sinewave* segment afterwards to measure $\Delta C_m$s. Recall that the maximum sweep length is limited to 16 ksamples. The high time resolution $C_m$ data for each sweep can be displayed in the *Oscilloscope* window for on-line evaluation of the progress of the experiment. *Auto CSlow* updates can be performed immediately before each sweep (*Update C-Slow before Sweep*) so that you will know if *R-series* is changing (e.g. if the pipette tip is becoming occluded) during the course of the experiment. This also ensures that capacitive current transients elicited by the depolarizing pulses are properly nulled.

ii) Low time resolution $C_m$ data can be acquired between pulses by using many sweeps that are linked together. The example in section 2.5. could be extended by linking a "Depol2" to "Sine2", followed by a "Sine3", then "Sine4", then "Depol3", etc... Each "Depol" series can then include a pulse to a different potential, or for a different duration. For example, if 5 different depolarizations are given, then 15 series are linked together. Just as in section 2.5., the "Sine" series are used to send points to XChart, whereas only the raw data from "Depol" sweeps are stored. This approach may be inelegant, but it works just fine. It has the further advantage that the different potentials or durations of the depolarizing pulses can be arranged in a scattered order.

## 2.7. Trains of depolarizing pulses

Here we see how to create trains of depolarizing pulses with short inter-pulse intervals to look at depolarization-induced changes in $C_m$ .

Very short inter-pulse intervals can be created by having a train correspond to a single sweep. In this case *constant* segments which produce depolarizing pulses are separated by *Sinewave* segments at *V-membr*. The maximum allowable number of points per sweep limits the number of pulses that can be applied without a gap. The other approach is to use multiple sweeps to produce a train. Whereas a virtually unlimited number of pulses can be given in a train, "gaps" in the $C_m$ record occur between pulses.

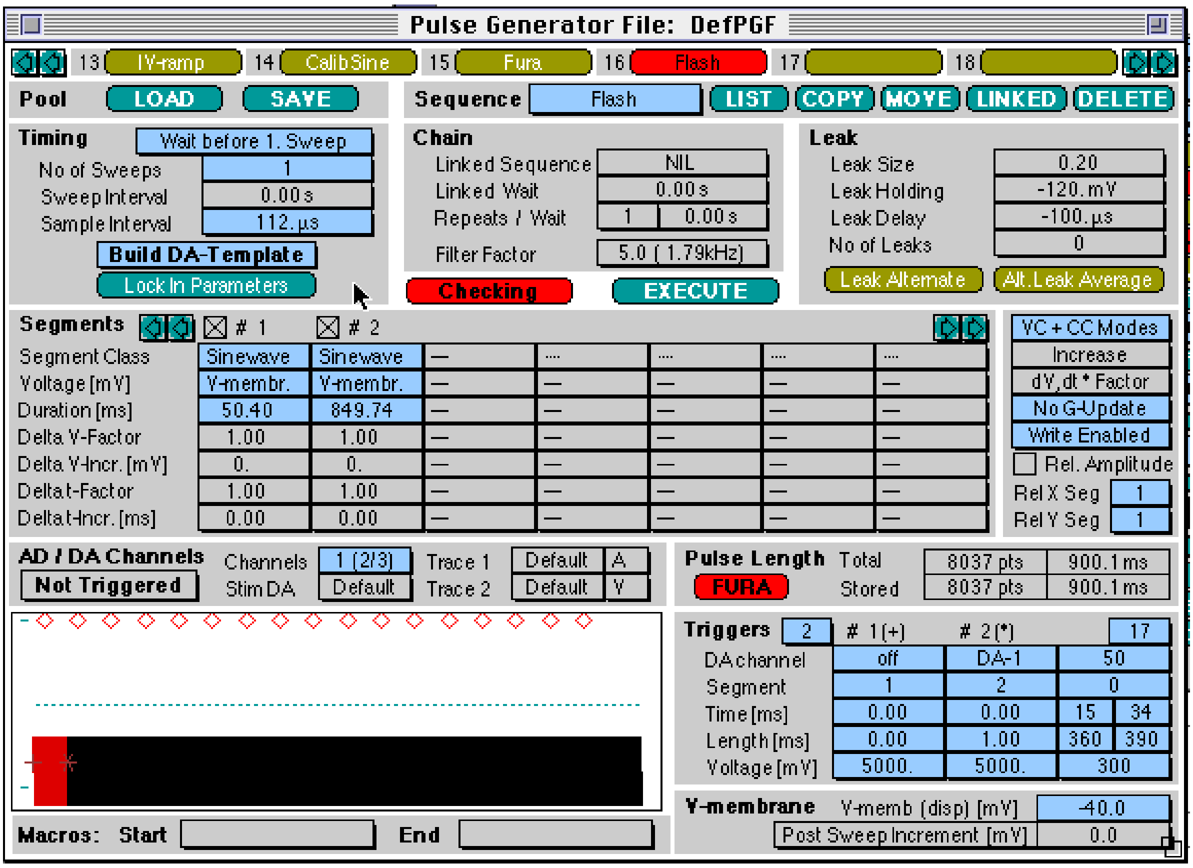
Example A. 50 ms pulses are applied with a 100 ms inter-pulse interval. A 1 kHz sine wave is used with 10 points/cycle for a total sampling rate of 10,000 points/s. The maximum number of points per sweep is 16k, corresponding to about 1.6 s. Thus about 10 pulses can be given in a single sweep. The series which produces this train is linked to series which provide low time resolution $C_m$ data to XChart (e.g. "Sine1" and "Sine2" in section 2.5.).

Example B. We wish to give 20 pulses of 50 ms duration, but are willing to tolerate a longer inter-pulse interval. Create a series with 20 sweeps with a leading *Sinewave* segment with a duration of 20 ms. This is followed by *Constant* segments defining the 50 ms depolarizing pulse followed by another *Sinewave* with a duration of 100 ms. The total inter-pulse interval is 120 ms plus the gap between sweeps. For a Quadra 800 writing to a RAM disk, the gap for this example is about 120 ms. Again, this series is linked to other series which provide low time resolution $C_m$ values during the inter-train interval.

## 2.8. Flash photolysis of caged $Ca^{2+}$. Multiple acquired inputs/outputs.

Flash photolysis of caged $Ca^{2+}$ to trigger rapid changes in $C_m$ presents an interesting example. Here, it is desirable for the software to trigger the flash lamp at a specific time in the sweep via a digital pulse output from one of the D/A outputs. It is also useful to concurrently sample a fluorescent signal which indicates the $Ca^{2+}$ time course immediately after the flash. Finally, in our example we will also have the software control a monochronomator through another D/A output to provide the dual wavelength excitation of the $Ca^{2+}$-sensitive dye. One should be warned that this example is quite complicated because of the demanding nature of the application. On the positive side, however, the example illustrates a few "advanced" topics in the use of *LockIn* with PULSE software.

A sample PGF for photolysis of caged $Ca^{2+}$ is given below:



Pulse Generator File: DefPGF

13 IV-ramp 14 Calib Sine 15 Fura 16 Flash 17 18

**Pool**

**Sequence** Flash

**Timing** Wait before 1. Sweep
No of Sweeps 1
Sweep Interval 0.00 s
Sample Interval 112. µs

**Chain**
Linked Sequence NIL
Linked Wait 0.00 s
Repeats / Wait 1 0.00 s
Filter Factor 5.0 ( 1.79kHz)

**Leak**
Leak Size 0.20
Leak Holding -120. mV
Leak Delay -100. µs
No of Leaks 0

**Segments** ☒ # 1 ☒ # 2

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| Segment Class | Sinewave | Sinewave | — | .... | .... | .... | .... |
| Voltage [mV] | V-membr. | V-membr. | — | — | — | — | — |
| Duration [ms] | 50.40 | 849.74 | — | — | — | — | — |
| Delta V-Factor | 1.00 | 1.00 | — | — | — | — | — |
| Delta V-Incr. [mV] | 0. | 0. | — | — | — | — | — |
| Delta t-Factor | 1.00 | 1.00 | — | — | — | — | — |
| Delta t-Incr. [ms] | 0.00 | 0.00 | — | — | — | — | — |

☐ Rel. Amplitude
Rel X Seg 1
Rel Y Seg 1

**AD / DA Channels** Channels 1 (2/3) Trace 1 Default A
Stim DA Default Trace 2 Default V

**Pulse Length** Total 8037 pts 900.1 ms
Stored 8037 pts 900.1 ms

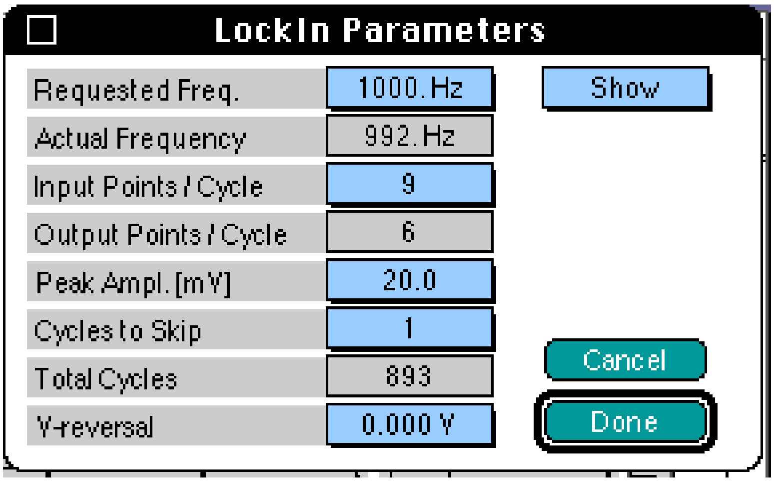
**Triggers** 2

| | # 1(+) | # 2(*) | 17 | |
|---|---|---|---|---|
| DA channel | off | DA-1 | 50 | |
| Segment | 1 | 2 | 0 | |
| Time [ms] | 0.00 | 0.00 | 15 | 34 |
| Length [ms] | 0.00 | 1.00 | 360 | 390 |
| Voltage [mV] | 5000. | 5000. | 300 | |

**V-membrane** V-memb (disp) [mV] -40.0
Post Sweep Increment [mV] 0.0

**Macros:** **Start** **End**

The following is the *LockIn Parameters* window for this PGF:



| | |
|---|---|
| Requested Freq. | 1000. Hz |
| Actual Frequency | 992. Hz |
| Input Points / Cycle | 9 |
| Output Points / Cycle | 6 |
| Peak Ampl. [mV] | 20.0 |
| Cycles to Skip | 1 |
| Total Cycles | 893 |
| V-reversal | 0.000 V |

- Why are there **2** *Sinewave* segments? The first segment precedes the flash trigger and serves to define the "baseline" $C_m$ value. If *Auto Scaling* is selected as the *Display Setting* in the *LockIn* configuration window, then the average $C_m$ value for the **first** *Sinewave* segment is determined and aligned to a grid line in the *Oscilloscope* window (see section 3.1.). This allows the user to quickly and easily determine the $\Delta C_m$ that results from the flash and automatically aligns the results from multiple flashes.

- There are 3 D/A outputs. i) The stimulus voltage. ii) Trigger number 2 initiates the flash through DA-1. iii) The monochronomator is controlled by the DA specified in the *Fura Configuration* window.

- There are 2 A/D inputs. i) Imon. ii) The fluorescence signal which is sampled by the AD specified in the *Fura Configuration* window.

- 2 triggers are selected. The DA channel of trigger #1 is selected as *off*, therefore this is a "logical" or software trigger. In PULSE, the first trigger defines the start of acquisition, so for our purposes this does not require an output signal. Trigger #2 initiates the flash with a 1 ms duration, 5 V amplitude pulse at the beginning of the second *Sinewave* segment. The column in place of trigger #3 actually specifies the parameters for controlling the monochronometer. See documentation of the FURA extension of PULSE for the meaning of these parameters.

- Since we have 2 inputs and 3 outputs, the number of *Output Points/Cycle* equals 2/3 the number of *Input Points/Cycle* (see section 3.2.1.). However, both numbers must be integers. Therefore we selected a value of "9" for the *Input Points/Cycle*, which results in a value of "6" for *Output Points/Cycle*. We set a *Requested Freq.* of 1000 Hz and got an *Actual Frequency* of 992 Hz.

- The maximum number of samples that can be acquired in a sweep is 16k. For 2 inputs, we get 8k per input channel. With 9 *Input Points/Cycle,* we can record 910 cycles, which corresponds to a total duration of 0.917 s. Thus we have set the duration of segment 2 to about 850 ms so that we can record for the maximum amount of time at high time resolution. We could link this series to another one to acquire more high time resolution points, but a gap will occur between sweeps.

## 2.9. Using *LockIn* with a patch-clamp amplifier other than an EPC9.

The following are important points to keep in mind when using *LockIn* with an amplifier other than an EPC9.

- *Measured Calibration* must be selected as the *Calibration Mode* in the *LockIn Configuration* window. Capacitance compensation must be switched *off* and the *C-Slow* parameter must be set to "0.0" in the *Configuration* window when a *Measured Calibration* is performed. As discussed in section 3.1**, any change of a "critical parameter"** (Table 1, e.g. frequency of sinusoid, external filter setting, stimulus time constant) **requires that a new *Measured Calibration* be made**. There is no way for *LockIn* to "know" if you change a "critical" parameter, so it is up to the user to ensure that the calibration remains valid.

- Use of series resistance compensation **is not** supported by the software and will produce erroneous results.

- Capacitance ("Cslow") compensation **is** supported. Compensated values (*C-Slow, G-Series*) are entered in the *Configuration* window (F11) as "Default" *Parameter* values. If capacitance compensation is *off*, enter a value of "0.0" for *C-Slow* (**not** for *G-Series*). If your patch-clamp amplifier is calibrated to give "R-Series" rather than *G-Series* values (e.g. Axopatch 200), you must invert the value and enter it as *G-Series*. **It is up to the user to ensure that *G-series* and *C-Slow* values always reflect the current settings of the patch-clamp amplifier.**

## 2.10. Exporting LockIn Data. Analysis of $C_m$ data in Igor by the "Depol" macro.

Analysis of *LockIn* data may vary considerably from user to user. Since it seems unlikely that "canned" analysis software would satisfy most users, the decision was made that *LockIn* data would be analyzed within Igor or some other general purpose analysis and display software. "Depol", an Igor macro for analysis of changes in $C_m$ elicited by sweeps containing a single depolarizing pulse, is available free of charge from HEKA. The macro can also integrate the current that flows during the depolarizing pulse, which is useful for estimating total $Ca^{2+}$ influx if the voltage-dependent currents are carried by $Ca^{2+}$. In this case the raw current data must be exported in addition to *LockIn* data. Users are free to modify and extend this macro to serve their own needs. The following example illustrates the export of *LockIn* traces and their subsequent analysis within Igor.

1) Execute the example in section 2.5. to create a number of stored series which consist of sweeps containing a single depolarizing pulse.

2) Select the *Replay* window and scroll through the stored series using the arrow keys. As individual series are highlighted, *Mark* those intended for further analysis by pressing 'M'. The series becomes dark as it is marked. All marked sweeps are processed at one time by "Depol". At least 2 sweeps must be marked for the macro to work properly.

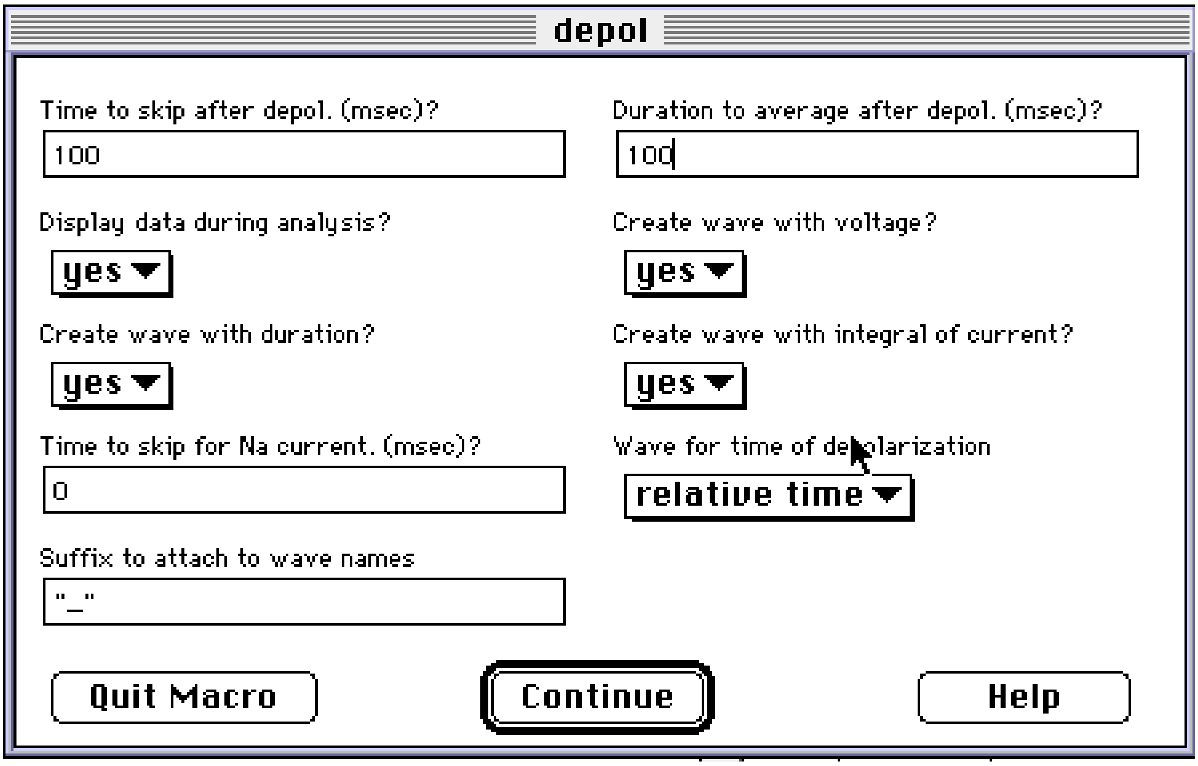
3) From the *Marks* pull-down menu, select *LockIn: Export to Igor.* The software then prompts for a *filename*.

A file with the name *filename.IGB* is created together with a folder named *filename Folder*. The folder contains 4 files for every exported sweep; three files correspond to Igor binary waves for $C_m$, $G_s$ and $G_m$ data whereas the fourth is a text file which contains critical information about the sweep which is read by "Depol". Double clicking on the *filename.IGB* file (preferably after quitting PULSE) starts Igor and loads **all** of the exported traces. However, we rarely use this *.IGB* file to load waves, and instead prefer using the macro.

4) If you want information about the integral of the $Ca^{2+}$ currents elicited by the depolarizing pulses, then you will need to export the raw current traces. Select *Export All* from the *Marks* pull-down menu. Again, you will be prompted for a filename. For "Depol" to work correctly, make sure the first 4 characters match the name you gave when exporting *LockIn* traces.

5) Exit PULSE and double click on the "Depol" procedure, which starts Igor.

6) Select *Depol* from the *Macros* pull-down menu of Igor. The following menu appears:


depol

Time to skip after depol. (msec)? 100

Duration to average after depol. (msec)? 100

Display data during analysis? yes

Create wave with voltage? yes

Create wave with duration? yes

Create wave with integral of current? yes

Time to skip for Na current. (msec)? 0

Wave for time of depolarization relative time

Suffix to attach to wave names "_"

The macro processes **all** $C_m$ sweeps that are contained in the exported folder. $G_m$ and $G_s$ traces are not used. The first *Sinewave* segment is averaged to provide the baseline $C_m$ value. $C_m$ values over a specified interval are averaged after the pulse to obtain a "post-pulse" $C_m$. The baseline value is then subtracted to get a $\Delta C_m$. The interval for averaging "post-pulse" $C_m$ can be selected sufficiently long after the pulse so that "gating artifacts" after the pulse are reduced (Horrigan and Bookman, 1994). In addition, if the interval is brief and placed shortly after the depolarization, then $\Delta C_m$ only indicates changes that occurred during the pulse. Placing the interval long after the pulse leads to $\Delta C_m$ values which include changes occurring after the pulse.

- *Time to skip after depol. (msec)?*

This specifies the delay in milliseconds from the beginning of the second Sinewave segment to the **start** of the interval over which $C_m$ values are averaged to determine a "post-pulse" $C_m$. We will use "100" (msec) for our example.

- *Duration to average after depol. (msec)?*

This specifies the duration of the "post-pulse" averaging interval. We will use a value of "100" for our example. $C_m$ values between 100 and 200 ms after the start of the second *Sinewave* segment will be averaged.

- *Display data during analysis?: yes/no*

If *yes* is selected, then each $C_m$ trace is displayed and lines are drawn to indicate baseline and post-pulse $C_m$ values. This allows the user to evaluate how validly the automated analysis of $\Delta C_m$ is proceeding. If the currents are integrated, then they are displayed also. A brief pause is inserted to give the user a look at the sweep before it is erased and replaced with the next sweep. Selecting *no* allows the macro to execute much faster. Select *yes* for our example.

- *Create wave with voltage?, Create wave with duration?: yes/no*

The macro can read the exported text file to get the voltage and duration of each depolarizing pulse. In order for these values to be correct, however, **the depolarizing segment must be specified as the *Rel Y Seg* in the PGF template**. If *yes* is selected, then Igor waves are created ("vwav_", "dwav_") which contain the pulse potentials and durations, respectively. For our example, select *yes* for both.

- *Create wave with integral of current?: yes/no*

*Yes* is selected if integration of the currents elicited by depolarization is desired. In this case, the raw current sweeps must have been exported (see above). Select *yes* for our example.

- *Time to skip for Na current (msec)?*

This is only applicable if integration of the currents is selected. The start of the integration interval can be delayed to allow the exclusion of inactivating $Na^+$ currents. **This time to skip, however, starts from the end of the first *Sinewave* segment.** Thus if you have a 5 ms *Constant* segment at *V-membr.* before the depolarizing segment, and you want to skip the first 5 ms of the depolarization-evoked current, then a value of "10" is appropriate. We will use a value of "0" for our example. In this case the current is integrated over the entire interval between *Sinwave* segments.

- *Wave for time of depolarization: relative time / absolute time*

A wave ("twav_") is created which indicates the time that each sweep was acquired. *Relative time* assigns a time of "0.0" to the first exported trace. *Absolute time* assigns values as the number of seconds from some arbitrary date (Jan. 1, 1904). Choose *relative time* for our example.

- *Suffix to attach to wave names*

In some situations it may be desirable to *Mark* and *Export* two or more different sets of sweeps from the same experiment. For example, "control" sweeps could be marked and exported to one folder whereas sweeps acquired after application of a drug would be exported to a different folder. "Depol" could then be run twice and the two sets of responses compared. In this case the waves created by "Depol" need to have different names for each analyzed set. Therefore a user-selectable suffix is attached to base wave names each time "Depol" is executed. **The macro requires that at least one character be selected as a suffix.** For our example, use the default character: "_".

7) Press *Continue* to start execution of the macro.

8) You will be prompted to select the folder with the exported *LockIn* waves. Navigate to highlight the folder, then press the *Folder* button.

9) If you selected *yes* for *create wave with integral of current?*, then you will be prompted to select the folder with the exported raw current traces.

You will now see the sweeps drawn to the screen and the pre- and post-$C_m$ values indicated by lines drawn on the traces. The currents are also displayed over the interval where they are integrated. Any *Constant* segments (e.g. at *V-membr.)* after the depolarizing *Constant* segment are encompassed in the integral, which allows "tail currents" to be included. Traces are not necessarily analyzed in the order that they were acquired, however, the final waves are sorted in correct sequential order.

In our example, "Depol" creates 6 waves: "**delCm_**" contains the $\Delta C_m$ values, "**twav_**" contains the times of the waves, "**qwav_**" contains the current integrals, "**dwav_**" contains the duration of each depolarization, and "**vwav_**" contains the potential of each depolarization. The $C_m$ and raw current waves are "killed" after analysis. This allows large data sets to be analyzed without running out of memory. You now have all of the power of Igor's graphics capabilities to present the results in any way you wish. For example, you can plot "delCm_" versus "twav_", "qwav_", "dwav_" or "vwav_".

## 2.11. Using *LockIn* in the *piecewise-linear* mode.

The piecewise-linear method is the most commonly used technique for measuring **changes** in membrane capacitance. Usually, CSlow compensation is used, which eliminates the bulk of the sinusoidal current which results from the sinusoidal voltage stimulus. The residual current is processed with a phase sensitive detector set to an appropriate phase angle to produce an output signal which is linearly proportional to small changes in $C_m$.

The most common way to set the phase is to induce a change in series resistance and then calculate the phase setting which produces an output which is insensitive to this change ("phase tracking", Fidler and Fernandez, 1989). This method, however, can produce phase errors because the pipette capacitance is neglected in the analysis (Gillis, 1995). Calibration is usually performed by offsetting *C-Slow* compensation by a defined amount (thus simulating a change in $C_m$) and noting the change in output of the phase sensitive detector.

*LockIn* avoids errors introduced by "phase tracking" by calculating the *PL Phase* from *C-Slow*, *G-Series* and an estimate of $G_m$ which is generated during *Auto CSlow* compensation (*Gmem*) according to:

$$PL\ Phase = 2\tan^{-1}(\omega Cslow / Gmem) - 2\tan^{-1}[\omega Cslow / (Gmem + Gseries)] - 180°$$

In principle, the use of this phase leads to a $\Delta C_m$ signal which is insensitive to changes in $G_s$ and only mildly sensitive to changes of $G_m$ under common recording conditions (Gillis, 1995; Joshi and Fernandez, 1988). If a patch-clamp amplifier other than an EPC9 is used, then *Gmem* is assumed to be zero. In this case the phase leads to a $\Delta C_m$ signal which is insensitive to changes in $G_m$ and mildly sensitive to changes of $G_s$ (Gillis, 1995; Joshi and Fernandez, 1988). Note that the use of a calculated phase requires that the phase shift introduced by the recording instrumentation be known. This is not a problem for *LockIn*, because phase calibration is an integral part of the software (see section 3.1.)

Calibration of the output traces is also automatically performed by *LockIn* using the formulas given by Neher and Marty (1982). Thus displacement of the *C-Slow* setting is not necessary for calibration, but can be used to test the calibration and phase setting as illustrated by the following example.

1) Follow steps 1) - 6) from the example in section 2.1. We will use the "Sine" series for our example.

2) Perform an *Auto C-Slow* compensation (press 'B'). We are going to offset the *C-Slow* setting for testing, therefore it is convenient to set *C-Slow* to a round number. Our value of *C-Slow* is 21.56 pF, so we reset it to 21.6 pF.

3) Open the *LockIn Configuration* window (<Comand><->) and toggle the *LockIn Mode* from *Sine + dc* to *Piecewise linear*. Type in "Sine" as the *Calib. Sequence*. Press the *Compute* button in the *PL-Phase* line. This automatically closes the *LockIn Configuration* window and prints the *PL-Phase* value to the *Notebook* window. The value should be approximately 284˚.

4) Execute the "Sine" series. In our case, the following values were printed to the *Notebook*:

```
A:  1.011 nS        B:  396.6 pS
dGM:1.605 nS        dGS:  -2.839 nS       dCM:  100.2 fF
```

5) Decrease the *C-Slow* setting by 0.1 pF (in our case to 21.50 pF) to simulate a **increase** in $C_m$. Execute the "Sine" series again. We got the following values:

```
A:  993.5 pS        B:  816.7 pS
dGM:  1.577 nS      dGS:  -2.79 nS        dCM:  206.4 fF
```

The difference in the "dCM" values is 106.2 fF, which indicates that there is about a 6% discrepancy between the calculated calibration of the $\Delta C_m$ trace and the calibration indicated by displacement of the *C-Slow* setting.

Ideally, no changes in the "dGS" or "dGM" values should result from the *C-Slow* offset. How do we evaluate if the actual changes suggest a problem in the setting of P-L Phase? The 'A' value represents the "Real" value of the conductance which remains after *C-Slow* compensation and with a rotation of axes set by P-L Phase. 'A' is multiplied by two different scaling factors to give the $\Delta G_s$ and $\Delta G_m$ values. The 'B' value is the corresponding "Imaginary" value which is scaled to give the $\Delta C_m$ value. Note that 'B' changes much more than 'A' upon displacement of the C-Slow value. A "phase discrepancy" between the calculated *P-L Phase* and the phase suggested by *C-Slow* displacement is given by: $\tan^{-1} (\Delta A / \Delta B)$. For our example, the phase discrepancy is -2.4°.

We can also test the PL mode by displacing the *RSeries* setting:

6) Set *RSeries* to a rounded value. In our case we used 5.6 MΩ. (Note that the value of *RSeries* indicated in the *EPC9* window is only displayed to 2 decimal places of precision. The actual value may be slightly different.) Execute "Sine". We got the following values:

A: 279.3 pS B: 386.1 pS
dGM: 447.0 pS dGS: -773.7 pS dCM: 98.33 fF

7) Now set *RSeries* to 5.5 MΩ and execute "Sine" again. We got:

A: 1.472 nS B: 441.7 pS
dGM: 2.355 nS dGS: -4.077 nS dCM: 112.5 fF

Note that "dCM" changed only slightly whereas "dGS" changed by -3.30 nS. The displacement of *RSeries* simulates a change in $G_s$ of -3.25 nS. Note that "dGM" changed by 1.91 nS. This represents the change in $G_m$ that would have to occur to result in the same change in admittance as the displacement in the *RSeries* setting. **In the *Piecewise-linear* mode, it is impossible to distinguish if a change in a "ΔG" trace originates from an actual change in $G_m$ or $G_s$.**

In most respects the *Piecewise-linear* mode can be used similarly as the *Sine + dc* mode as illustrated by the examples in the previous sections. Values labeled as "Cm", "Gm" and "Gs" (such as in XChart) should now be interpreted as "ΔCm", "ΔGm" and "ΔGs". In order for the *P-L Phase* to remain valid, it is important to perform *Auto CSlow* updates followed by execution of *Compute P-L Phase* periodically (say, once per minute). **Both of these operations can result in jumps in the XChart ΔCm trace which are unrelated to exocytosis or endocytosis.**

# 3. Parameter Settings of *LockIn*

## 3.1. The *LockIn Configuration* Window

This window is selected from the *Pulse* pull-down menu or by pressing <Comand><->.

- *LockIn Mode: Sine + dc* or *Piecewise linear*

These two modes of operation are explained in section 1.1. ***Sine + dc* is the recommended mode**. The tutorials in sections 2.1 - 2.10 use the *Sine + dc* mode. Section 2.11 illustrates the use of the *Piecewise linear* mode.

- *Calibration Mode: Calculated* or *Measured*

As mentioned in the introduction, one of the great advantages of *LockIn* when used in sine + dc mode is that equivalent circuit parameters are estimated using measurements of the total admittance, not just relative changes. True admittance measurements require that the absolute phase shift between the stimulus voltage sinusoid and the resulting sinusoidal current be determined. This, in turn, means that **the phase shift (y) and attenuation (a) introduced by the patch-clamp amplifier as well as all low-pass filters present in the stimulus or Imon pathways be accounted for.** There are two ways that 'y' and 'a' can be determined with *LockIn*: *Measured Calibration* and *Calculated Calibration*.

*Measured Calibration*

With this form of calibration, a resistor is inserted in the head stage and a sine wave series is executed. Since an ideal resistor introduces zero phase shift between applied voltage and measured current, 'y' is readily measured. The value of the resistor is measured at dc as simply the average *Imon* value divided by *V-membrane* (note that **this assumes that *Vo*ffset is set correctly**). This "true" value of resistance, together with the ac measurement leads to an estimate of 'a'. 'y' and 'a' are stored with each acquired sweep as well as in the Configuration file of PULSE. Note that the estimate of 'y' and 'a' are only valid for a particular setting of the "critical parameters" (Table 1). **Any change of a "critical parameters" (e.g. frequency of sinusoid, Filter 2 setting) requires that a new *Measured Calibration* be made**. The settings of the "critical parameters" that were used for the calibration are also stored in the Configuration file of PULSE, so, if an EPC9 is used, a change in a "critical parameter" will be recognized by *LockIn* and an error message will be generated.

*Performing a Measured Calibration*

1) Create a basic series (we will call it "Sine") with values for *Actual Frequency* and *Output Points/Cycle* (which is a function of *Input Points/Cycle* and the number of inputs and outputs, see section 3.2.1.) that you will use for all your experimental series. Specify a *Sinewave* segment with a duration of at least 100 ms. The *Peak Ampl.* of the sine wave should be something like 20 mV (1

mV if you are calibrating for the high gain range: ≥ 50 mV/pA).

2) In the *EPC9 Patch-Clamp* window, set *Filter 1, Filter 2, Stim* time constant, and *gain* range to values that you will use during acquisition. CSlow compensation should be *Off*.

3) We have found that the 10 MΩ resistor built into the MC-9 model circuit works fine for calibration for frequencies up to about 2 kHz. Attach the MC-9 to the head stage.

4) Set the switch to the middle position and perform an *Auto CFast* compensation (or press 'A'). Then set the switch to the "10 M" position.

5) Perform an *Auto V*offset compensation (or press Z). Then set *V-membrane* to something like -20 mV (-1 mV if you are calibrating for the high gain range: ≥ 50 mV/pA).

6) Open the *LockIn Configuration* window from the *Pulse* pull down menu (or press <Comand><->) and set the *Calibration Mode* to *Measured*. The *LockIn Mode* should be *Sine + dc*. Make sure the *Write to Notebook* box is checked. Type in the name of your test series ("Sine") in the *Calib. Sequence* box.

7) Press the *Perform Measured Calibration* button.

The Phase and attenuation that were found are printed to the Notebook window. Now you can switch the MC-9 to "0.5 G" and execute the "Sine" series. The values sent to the Notebook window should be something like RS: 5.2 MΩ RM: 510 MΩ CM: 22 pF.

### *Calculated Calibration*

This type of calibration is a feature unique to *LockIn* software and EPC9 hardware combination. With this combination, all "critical parameters" are under software control and it is possible, in principle, to **predict** the phase shift and attenuation introduced by the instrumentation. A software model of the EPC9 has been constructed which is quite accurate in predicting 'y' and 'a' under almost all experimental situations. The phase is generally accurate within a couple of degrees (usually within 1 degree) and the attenuation is reliable within a couple of percent. **Recent versions of *LockIn* ( > 8.06) have significantly improved the accuracy of Calculated Calibration**. It is recommended that you obtain from HEKA the latest version of PULSE. When a "critical parameter" is changed, *LockIn* automatically recalculates the phase and attenuation, leading to great flexibility in the execution of experiments.

### *Which Calibration Mode should I use?*

*Calculated Calibration* provides the most flexibility since changes in "critical parameters" can be made without performing a new calibration with an external resistor. *Measured Calibration* can tweak the software to get the last degree of accuracy, *assuming the resistor used behaves ideally*. This mode can also be used to enforce logical choices for the "critical parameters". If you perform an *External Calibration* with a well thought out set of "critical parameters", then the software will warn you if you inadvertently change, for example, the *Filter 2* setting. Finally, *External Calibration* **must** be used for amplifiers other than the EPC9, or if you use external low pass filters in the stimulus or Imon pathways.

- *Phase Shift*

This user can specify a phase shift to be applied to the "residual" admittance before the equivalent circuit parameters are calculated. This phase shift is only applied to the actual current that is measured and not the component of admittance which is "nulled out" by CSlow compensation. This feature can be used, for example, to "tweak" the phase determined by *Measured* or *Calculated Calibration*. Another example where this would be useful is if data are recorded with a set of "critical parameters" which differs from those that were used when a *Measured Calibration* was performed (this is prevented by the software if an EPC9 is used, but can occur for other amplifiers). The phase difference can be determined by performing a new *Measured Calibration* and can be specified as the *Phase Shift* for *Replay* of the recorded data. G**enerally the *Phase Shift* should be left at 0.0°**.

- *PL Phase / Compute*

These settings are only applicable if *Piecewise linear* mode is used. *PL Phase* is the setting of the phase sensitive detector (lock-in amplifier) after correction for shifts introduced in the instrumentation (see *Calibration Mode*). Ideally, this phase setting results in a $C_m$ output which is maximally sensitive to **changes** in capacitance while insensitive to changes in $G_s$ or $G_m$. The value can be entered manually or is **calculated** from the current *C-Slow* and *G-Series* values by pressing the *Compute* button. This calculated method of determining the *PL Phase* differs fundamentally from (and avoids errors associated with, see Gillis 1995) empirical determinations using series resistance dithering ("phase tracking", Fidler and Fernandez, 1989). It is more closely related to finding the phase empirically via "CSlow dithering" (Neher and Marty, 1981) but, just like the *sine + dc* mode, requires the phase shift introduced by the instrumentation to be known. The PL Phase is stored with each sweep that is acquired in *Piecewise linear* mode. See section 2.11. for an illustration of the *Piecewise linear* mode.

- *Calib. Sequence*

Here the user specifies the series to be used to *Perform Measured Calibration* or to *Compute PL-Phase*.

- *Perform Measured Calibration*

Only applicable for *Calibration Mode: Measured*. The user presses this button to perform a *Measured Calibration*. See "Performing a measured calibration" earlier in this section.

- *Write to Notebook*

Checking this box causes average $C_m$, $R_s$ and $R_m$ values for each sweep to be sent to the *Notebook* window.

- *Display Settings: Auto Scaling* or *Fixed Scaling*

This setting controls the scaling for high time resolution display of $C_m$ values in the *Oscilloscope* window. *Auto Scaling* is usually much more practical and is therefore recommended. In order for $C_m$ values to be displayed, 1) the *Display Mode* must be set to *I vs. t + LockIn* in the *Display* pull-down menu and 2) *Show* must be selected in the *LockIn Parameters* window of the series to be executed.

*Auto Scaling*

With this setting, the average $C_m$ value for the **first** *Sinewave* segment is determined and aligned to a grid line. It is implicitly assumed that the first segment establishes a baseline $C_m$ before a depolarization is given or photolysis of caged $Ca^{2+}$ is triggered. (It is recommended that grids be turned on by selecting *Labeling: Grid + Labels* or *Grid + Values* in the *Display* pull-down menu).

*From: bottom, middle* or *top*

This determines which grid line is used for "baseline" $C_m$ (actually, the average of the first *Sinewave* segment). ***bottom*** is actually one grid up from the bottom-most grid. If increases in $C_m$ are expected to result during the sweep (the most typical case), then this setting allows the entire *Oscilloscope* window to be used most efficiently. Likewise, ***middle*** selects the middle grid and ***top*** selects the grid one down from the top-most grid.

*Scaling/Div.*

This determines the scaling per division (or grid) of the $C_m$ display in the *Oscilloscope* window when *Auto Scaling* is selected.

*Fixed Scaling*

Here, the bottom-most grid of the *Oscilloscope* window is assigned the value entered under *Display Y-min* and the top-most grid is assigned the value entered under *Display Y-max*.

- *Connect Markers*

This entry determines which type of markers (types: *Point, Plus, Star, Diamond, Cross, Square*) are used for $C_m$ points in the *Oscilloscope* window. If the check-box is checked, then lines are drawn between the symbols.

## 3.2. The *Pulse Generator* Window

The *Pulse Generator* window is selected from the *Pulse* pull-down menu or by pressing F12.

- *Segment Class: Sinewave*

Just as the name suggests, this segment class outputs sine waves with characteristics defined in the *LockIn Parameters* window. Note that all sine waves in a series must have the same *LockIn Parameters*. The dc potential of the sine wave signal is specified by *Voltage [mV]*. Once a *Sinewave* segment is specified in a sweep, **all** segment *Durations* must be integral multiples of the sine wave period. This is automatically taken care of by the software.

### 3.2.1. The *LockIn Parameters* Window

This window is opened by pressing the *LockIn Parameters* button in the *Pulse Generator* window.

- *Requested Freq.*

Here the user enters the desired frequency of the sinusoid in Hz. This value is not stored; once the *LockIn Parameters* window is closed, the requested frequency is set to the *Actual Frequency*.

- *Actual Frequency*

Only certain frequencies are possible because they are generated by dividing a fundamental clock frequency by an integer. Nevertheless, the *Actual Frequency* is usually within a couple of percent of the *Requested Frequency*. The *Actual Frequency* is a function of the *Requested Freq.*, the *Input Points/Cycle* and the number of input channels acquired.

- *Input Points/Cycle* ($p_i$)

The number of points per sine wave cycle which are sampled and processed to result in a single estimate of the equivalent circuit parameters ($C_m$, $G_s$, $G_m$). The sampling rate of Imon ('$f_s$') is given by the product: $p_i$**Actual Frequency*. Equivalently, the *Sample Interval* is the inverse of the product $p_i$**Actual Frequency*. It is recommended that '$p_i$' be set to a value of 8 or greater in order to reduce the "aliasing noise" of $C_m$ estimates (see Gillis, 1995, for details).

- *Output Points/Cycle* ($p_o$)

The number of points per sine wave cycle which are output by the stimulus DAC in constructing the sine wave. This value is not user-selectable, but calculated from '$p_i$' and the total number of input and output channels (see below). The "sine wave" output by the DAC actually has a "staircase" appearance because discrete output steps are used. A larger value of '$p_o$' leads to a sine wave with a smoother appearance (i.e. with smaller steps), however, Fourier analysis tells us that the desired sine wave is just as validly present in the crude signal that results from low values of '$p_o$'.

**How can '$p_o$' be different than '$p_i$'?**

The relationship between '$p_o$' and '$p_i$' is complicated because of the way the ITC-16 interface inputs and outputs values. Sampling of inputs and changes in output values are made at the same fundamental "cumulative sampling rate" ($f_{cum}$). With multiple input channels ('$n_i$' in number), each channel is sampled **consecutively**, not simultaneously. Thus '$f_{cum}$' is set to the product of '$n_i$' and the individual channel sampling rate ('$f_s$', the inverse of the *Sample Interval* set in the PGF window). If '$n_o$' is the number of output channels, then the output rate per channel ('$f_o$') is given by:

$$f_o = f_{cum} / n_o = \frac{n_i}{n_o} f_s :$$

Given this relationship, it is straightforward to show that:

$$p_o = \frac{n_i}{n_o} p_i$$

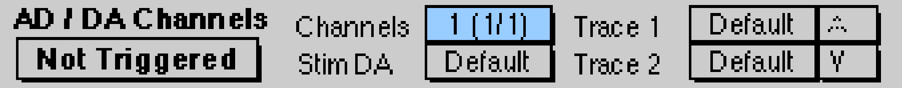
In the present version of PULSE, the user can select two AD channels for high speed sampling in the PGF window (one is normally Imon). However, with the FURA extension, an additional AD channel is possible, so '$n_i$' is in the range of 1-3. In addition to the Vstim output, 3 triggers are available as DA output channels (the output channel for the FURA extension, if selected, occupies *Trigger # 3*), therefore '$n_o$' is in the range of 1-4. If the DA channel for a particular trigger is set to *off*, then it is only a "software trigger" and doesn't count towards '$n_o$'. In addition, if the FURA extension is selected and the AD channel which is used for FURA acquisition is also selected as *Trace 1* or *Trace 2* (in order to save the high time resolution fluorescence signal), then the software "knows" not to count this channel twice in determining '$n_i$'.

Since the software keeps track of '$n_i$' and '$n_o$' and sets '$p_o$', why should the user care about these parameters? Two issues are of concern. i) '$p_o$' must be an integer. For example, if there are 2 inputs and 3 outputs, and you select 10 *Input Points/Cycle*, then you get the following error message: *The output points/cycle is not an integer number, do you want to adjust it?* If you select *Adjust It*, then (for this example) '$p_i$' will be set to 9 and '$p_o$' will be 6. Selecting

*Cancel* nullifies the selection you made that led to the error message. ii) The maximum **total** number of samples per sweep is limited to 16 ksamples, so having more than one input channel reduces the duration that you measure $C_m$ at high time resolution without a gap.

**Example:** A 1 kHz sinusoid with 10 *Input Points/Cycle* is desired. There are 2 inputs and 4 outputs, therefore *Output Points/Cycle* is 5. The maximum number of input points per channel is 16k/2. Therefore, the maximum number of sine wave cycles is 8k/10, corresponding to a total duration of slightly greater than 800 ms.

There is a quick way to find out how many inputs and outputs you have selected. You may have noticed a new set of numbers in the *Channels* box of the *PGF* window. The values in parentheses indicate the number of inputs and outputs: ($n_i/n_o$).



Channels 1 (1/1)
Stim DA Default
Trace 1 Default
Trace 2 Default

- *Peak Ampl. [mV]*

The amplitude of the sinusoid in millivolts. **Note that the peak-to-peak amplitude is twice this value.**

- *Cycles to Skip*

When a *Sinewave* segment begins, there is a "capacitive" transient current response just as there is a transient response when a voltage step is given. In order to prevent this from causing an artifact in the $C_m$ trace, sine wave cycles are skipped at the beginning of each *Sinewave* segment. If the frequency of the sine wave is chosen appropriately, then the transient should decay within a single cycle, **therefore a value of 1 is recommended**.

- *Total Cycles*

This indicates how many sine wave cycles occur in the entire sweep.

- *V-reversal*

Applicable in the *Sine + dc* mode. Since the dc current is used in processing estimates of the equivalent circuit parameters, the reversal potential must be known (see Figure 1 and Gillis, 1995). For our purposes, *V-reversal* is the zero current potential **extrapolated** from the slope conductance about *V-membr.*, which is not necessarily the same as the actual zero current potential. If you expect a membrane conductance to be activated during the course of the experiment, set *V-reversal* to the zero current potential of the activated conductance. This setting is actually not very critical if $G_m$ is low. **A value of zero is often used** in the common situation where $G_m$ is low and the actual reversal potential is unknown.

- *No Compute; Show; No Show*

This parameter allows controls of the high time resolution display of $C_m$ values in the *Oscilloscope* window at the level of an individual series.

*No Compute*

This selection disables all of the on-line computations of *LockIn* in order to speed up acquisition. This is only useful if the series is *Write Enabled* and *No Compute* is changed to *Show* or *No Show* during *Replay* of data.

*Show*

If *Show* is selected and if *Display Mode: I vs. t + LockIn* is selected in the *Display* pull-down menu, then the high time resolution $C_m$ trace is displayed in the *Oscilloscope* window for this series.

*No Show*

Prevents the display for this series of the $C_m$ trace in the *Oscilloscope* window.

## 3.3. The *Edit Traces and Parameters* window of XChart

This window is opened by selecting *Edit Traces and Param.* from the *X-Chart* pull down menu (or press <Comand><4>). See section 2.3 for an example of how to set up this window.

- *Type: LockIn*

Selection of this type allows the six possible types of *LockIn* values listed below to be sent to XChart.

- *Arg. 1: Real; Imag.; DC-Value; Cm; Gm; Gs*

Selection of the type of *LockIn* data to be acquired. *Real, Imag.* are the real and imaginary components of the admittance measurement. *DC-Value* is the conductance given by $I_{dc}$/ (*V-membr. - V-reversal*). *Cm, Gm*, and *Gs* are defined from Figure 1 and are calculated from the above three parameters.

## 3.4. Exporting *LockIn* data from the *Marks* pull-down menu

Low time resolution *LockIn* data acquired by the XChart extension can be exported for analysis using the Export command from the *X-Chart* pull-down menu. Export of high time resolution data occurs for sweeps which are marked in the *Replay* window. See section 2.10. for an example of export and analysis of high time resolution sweeps.

- *LockIn: Export to Igor*

When this is selected, the software prompts for a *filename*. A file with the name *filename.IGB* is created together with a folder named *filename Folder*. The folder contains 4 files for every exported sweep; three files correspond to Igor binary waves for $C_m$, $G_s$ and $G_m$ data whereas the fourth is a text file which contains critical information about the sweep which can be read by an Igor macro such as "Depol". Double clicking on the *filename.IGB* file starts Igor and loads and displays **all** of the exported traces. In many circumstances, it is preferable to load waves one at a time with a macro such as "Depol".

- *LockIn: Export to ASCII*

This creates an ASCII file with high time resolution *LockIn* information that can be read by any display and analysis program.

# 4. Tips on the use of *LockIn*

## 4.1. Recommended parameter settings for LockIn measurements

- *Frequency of the sinusoid ($f_c$).* The $C_m$ noise increases as $f_c$ exceeds the "break frequency" ($f_b$) defined as: $[2\pi C_m/(G_s+G_m)]^{-1}$. Very low values of $f_c$ (say, below $f_b/4$) also result in noisy $C_m$ estimates, see Gillis (1995) for details. Due to the non-ideal nature of $C_m$ estimation, different stimulus frequencies can lead to slightly different values of $C_m$. Therefore, once a value of $f_c$ is selected, it is best not to change it during the course of the experiment (i.e. all series used should have the same *Actual Frequency* in the *LockIn Parameters* window).

- *Number of input points per sinusoid.* Generally, a value of 8 or great is advised in order to minimize "aliasing noise" of $C_m$ estimates (see Gillis, 1995 for details). However, large values will reduce the maximum sweep duration, which is limited to 16 ksamples. If long sweeps are desired, 8 points/cycle is sufficient if Filter2 is set to twice the frequency of the sinusoid.

- *Amplitude of the sinusoid.* Larger amplitudes result in lower $C_m$ noise, but the activation of voltage-dependent conductances in excitable cells should be avoided. For excitable cells, setting *V-membr.* as hyperpolarized as the cell will tolerate allows a larger amplitude to be used. Very small amplitudes (say, below 2 mV) can cause "quantization problems", particularly if the number of output points/cycle is small.

- *V-reversal.* A value of "0.0" can be used if $G_m$ is small and the actual reversal potential is unknown. If you expect a significant membrane conductance to be activated during the course of the experiment, set *V-reversal* to the zero current potential of the activated conductance. In principle, this value can be found during pilot experiments by: i) stepping the membrane potential by about +/- 20 mV about *V-membrane* ii) measuring the steady-state currents at the two potentials and iii) extrapolating these values to the zero current potential.

- *Gain of the patch-clamp amplifier.* In principle, the larger value of feedback resistor used in the high gain range (≥ 50 mV/pA in the EPC-7/9) results in lower $C_m$ noise. However, the improvement is often negligible for "typical" circuit parameters and sinusoidal frequencies (Gillis, 1995). High gain makes *Measured Calibration* difficult and can easily lead to amplifier saturation. Typically of gain of 1-10 mV/pA is used.

- *Filter 1 setting (EPC9).* The *10 kHz Bessel* filter is usually an appropriate setting.

- *Filter 2 setting (EPC9).* A setting of (at least) twice the stimulus frequency ensures that estimates generated for each sinusoidal cycle are independent. Higher values than this can lead to "aliasing noise" unless the number of input points/cycle is high. The *Bessel* filter type is preferable if *Calculated Calibration* is used because the circuitry appears to follow the theoretical characteristics better than the *Butterworth* filter type.

- *Stimulus filtering.* It has been customary with software lock-in amplifiers to filter the stimulus D/A signal to produce a sine wave with a "smooth" appearance (i.e. remove the harmonics). However, this is not actually necessary, because the harmonics are automatically removed by the software lock-in algorithm. The 20 µs time constant filter built into the EPC-7/9 sufficiently smoothes the D/A signal to prevent amplifier saturation.

- *Capacitance Compensation.* The use of capacitance compensation is quite convenient the eliminate the current transients which result from depolarizing steps. However, there is an additional benefit. $C_m$ noise appears to be slightly less when *C-Slow* compensation in used in an EPC9, presumably because it cancels some of the noise present in the stimulus pathway (Gillis, 1995). Testing of *LockIn* is best performed with capacitance compensation *off*, because *LockIn* estimates are essentially identical to *C-Slow* and *R-Series* values when the bulk of the sinusoidal current has been nulled out. See section 2.1.

- *Series Resistance Compensation.* The use of series resistance compensation can actually reduce $C_m$ noise if a value of $f_c$ is used which approaches or exceeds the "break frequency" ($f_b$) defined above. This is because the circuitry will "boost" the amplitude of the stimulus sinusoid to partially compensate for the drop in voltage across $G_s$. Since a component of the current monitor signal is fed back to the stimulus, however, the $C_m$ trace can become noisier if a high percentage of *RSeries* compensation is used. The effect of series resistance compensation on the current signal is accounted for by *LockIn* when an EPC9 is used. This correction, however, is inexact -- particularly for large fractional compensation. Perhaps it is best that series resistance compensation only be used when it is needed.

## 4.2. Miscellaneous Tips

- *Calibrate the patch-clamp amplifier gain with an external resistor* (EPC9). The automatic calibration of the EPC9 performed by E9Screen is not exact. The gain can be "fine tuned" with an external resistor using a new feature of the E9Screen program. The 10 MΩ resistor in the MC-9 model circuit can be used since it is accurate to within 1%. The corrections are made in an *External Gain Calibration* table in the *Calibrate* pull-down menu of E9Screen and result in a change in the *SCALE.EPC* file. The updated file must be copied to the Pulse folder.

- *Reducing gaps between sweeps.* Sections 2.3. - 2.7. give examples where repeated sweeps of sine waves are given to result in low time resolution measurements of $C_m$ (one averaged $C_m$ point per sweep) which are sent to XChart for storage and display. Reducing the gaps which inevitably occur between sweeps allows a faster acquisition rate. One important way to reduce these gaps is to use multiple sweeps within a series instead of series repeats to produce the desired number of sweeps. This approach is illustrated by the example in section 2.5. Writing data to a **RAM disk** dramatically reduces the gap that occurs following a series that is *Write Enabled*.

- *Choosing the duration of sine wave sweeps used for low time resolution recording to XChart.* A longer duration sweep results in lower noise $C_m$ estimates because more sine wave cycles are included in the average. Shorter duration sweeps allow a higher rate of $C_m$ measurements, but this rate is ultimately limited by the gaps that occur between sweeps. A reasonable compromise between acquisition rate and noise is to make the sweep duration approximately equal to the gaps (i.e. use a 50% duty cycle). This is the strategy used in section 2.5.

- *Decimation of high time resolution $C_m$ values.* High time resolution estimation of $C_m$ occurs at the maximum rate that generates independent values -- one point per cycle. Estimates generated at this rate, however, tend to be noisy. Often the user is willing to trade off some of the time resolution for lower noise estimates. This can be done by "decimating" the data, i.e. average the data trace a given number of points at a time. This can easily be done within Igor. A *Decimation* procedure is defined in the *Analysis* folder within Wavemetric's *Procedure* folder.

## 5. References

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## Table 1. Parameters which affect admittance measurements

### 1) Scaling parameters

- Gain (trans-impedance) of the patch-clamp amplifier

- Amplitude of sine wave stimulus

### 2) "Critical parameters" which determine phase shifts (delays) and attenuation introduced by the patch-clamp amplifier and the sine wave generator

- Number of points/cycle output by the DAC in generating the sine wave

- Sine wave frequency

- Stimulus filtering (EPC-7/9: $t_s$ = 2 µs or 20 µs )

- Low-Pass filters present in the current monitor pathway. For the EPC9, this consists of:

    - Filter 1 ($F_1$ = 10 kHz Bessel, 30 kHz Bessel, HQ 30 kHz, or 100 kHz Bessel)

    - Filter 2 ($F_2$ = Bandwidth + Bessel or Butterworth)

- Feedback resistor of the patch-clamp amplifier head stage (For the EPC9: $R_f$ = 5 MΩ for low gain range; 500 MΩ for medium range; 50 GΩ for high gain range)

### 3) "Compensation" parameters

- Capacitance compensation (*C-Slow, G-Series*)

- Series resistance compensation. *LockIn* only supports this for EPC9 amplifiers.

- Leak subtraction (software or hardware). *LockIn* does not support hardware leak subtraction.

Figure 1: Admittance measurements with the EPC9 patch-clamp amplifier