**Protocol: Noise Analysis**

The use of noise analysis is typically needed in electrophysiology experiments when translocation or blockage times are smaller than the sampling rate of the instrument being used. Noise analysis cannot be used under all circumstances and caution must be taken in interpreting results. (See assumptions below.)

Assumptions: There are two major basic assumptions that are made when using simple noise analysis: that the system under study is a two-state system and that the system is a Markov process. A two-state process is one in which there are only two possible states that the system can be in (example: fully blocked/fully unblocked). A Markov process is one that has no memory; that is, the state of the system does not depend on those states preceding it (example: that a channel was blocked one second ago does not influence whether it is blocked now.)

Procedure for finding τ_b as a function of Voltage: The following procedure can be used to find average residence times of an analyte in a single channel. The dependence of this residence time on applied voltage can be found.

Data Acquisition should be preformed at with a Butterworth filter and standard single channel equipment (see diagram.)
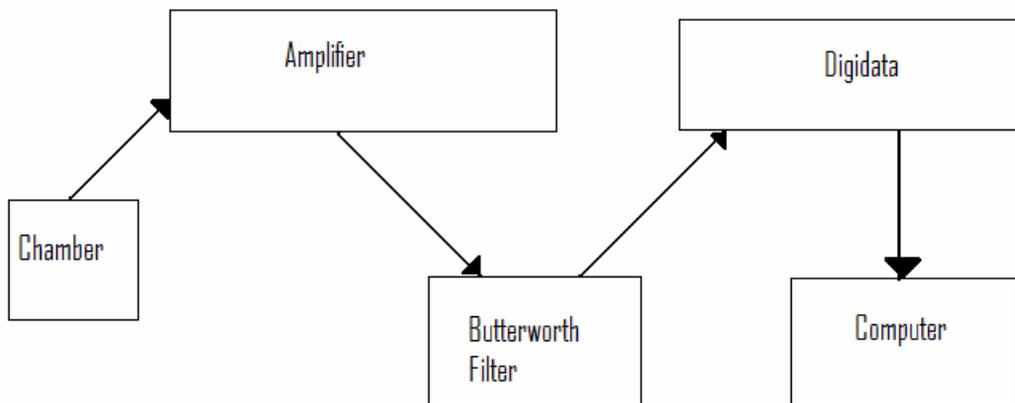
Acquire data with the Butterworth frequency set at 50kHz and the Digidata frequency set at 200kHz. (explanation below)
> In general, we want to minimize our time resolution, since this will increase the precision of our estimates. The sampling time interval is determined by frequency of the Butterworth filter feeding into the Digidata, and is given by the

inverse of this filter frequency. So we use the highest frequency available on the Butterworth Filter; in our case this is 50kHz. This gives a sampling time interval is 20μs. One must also set the acquisition time of the Digidata machine. **Note that this must be *at least* 2.5 times the Butterworth filtering frequency.** The sampling rate of the Digidata is set in Clampex software by going to Aquire→New Protocal. In our case a frequency of 200kV is appropriate.

Current traces should be attained at a range of several different positive and negative applied voltages. The voltage can be set using the Axon 200B. Traces of this type should be made for several different concentrations of analytes (as well as the channel without analytes.)

Analysis: Given that the process is two-state and Markov, theory predicts that the Power spectrum density of the current trace with respect to time should conform to this equation,

$$\tau_b = \frac{S_I(0)}{4\langle\Delta I\rangle\Delta i}\frac{1}{(1-\langle\Delta I\rangle/\Delta i)^2}$$

Where,
τ_b is the average time of blockage:
$\langle\Delta I\rangle$ is the average current drop,
$S_I(f)$ is the power spectral density of the current trace as a function of frequency with
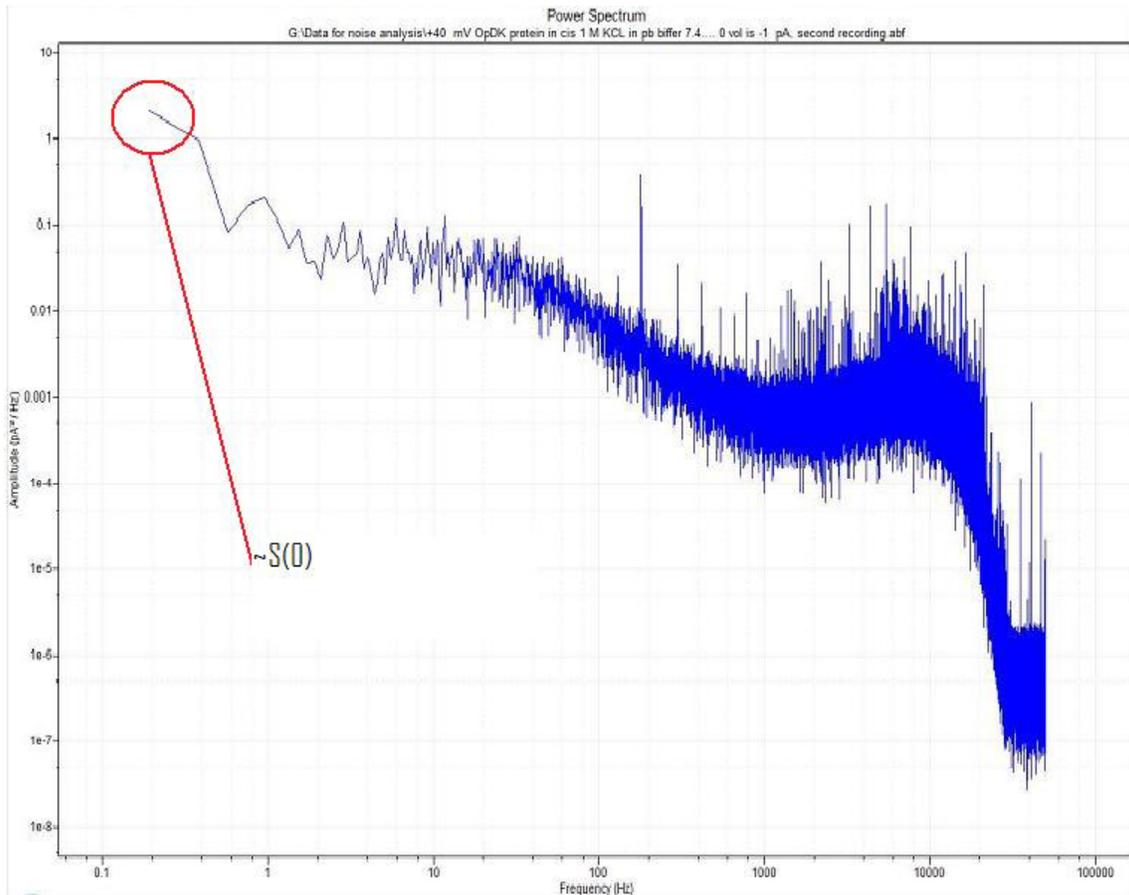$S_I(0)$ limit of the power spectral density as the frequency goes to zero.
Δi is the current drop due to a current blockage.

This formula can be used to find the residence time for the analyte in the channel at a given applied voltage and concentration.

To find $\langle\Delta I\rangle$, compare the average value of the current in a trace with added analytes to the average of the current trace without analytes $\langle I_{without}\rangle - \langle I_{with}\rangle$.
$\langle I\rangle$ for a trace can be found by using Clampfit. When the current trace is in an open window, select the region you wish to analyze with cursors then go to Analyze→Histogram. Select the appropriate cursors. Press OK. You may have to adjust the bin number if the resulting histogram does not look Gaussian. When the Histogram window opens, go to Analyze→Fit and select "Gaussian" under predefined function. This should result in a window that looks like the one below.

The average current can be read from by selecting Analyze→Fitting Results.

The Power Spectral Density $S_I(f)$ can be found in ClampFit. When the current trace is in an open window, go to Analyze—>Power Spectrum
Leave "Type" as "None"
Check the box that says "Average spectral segments"

$S_I(0)$ can be determined from the lower limit of the frequency of the Power spectral density of the current trace.

Δi is not measured directly when the time sampling rate is longer than the transitions. If we can assume that the process remains two-state and Markovian at high concentrations then we may take the limit of the current drop as the concentration goes to infinity (that is when the channel is always blocked with the analyte).
To do so, one must make several current traces with incremental increases in analyte concentration. (i.e. the experiment must be repeated several times at higher concentrations)
Plot the average current in each trace $\langle I \rangle$ (as found above) and subtract this from the average current in the trace with no analytes, against concentration. The observed curve should approach a value at high concentrations. This can be done in Excel.

$Current_i - Current_{base}$	Concentration
$\langle I \rangle_{base} - \langle I \rangle_{base}$	0
$\langle I \rangle_1 - \langle I \rangle_{base}$	1
$\langle I \rangle_2 - \langle I \rangle_{base}$	2
$\langle I \rangle_3 - \langle I \rangle_{base}$	3
$\langle I \rangle_4 - \langle I \rangle_{base}$	4
$\langle I \rangle_5 - \langle I \rangle_{base}$	5
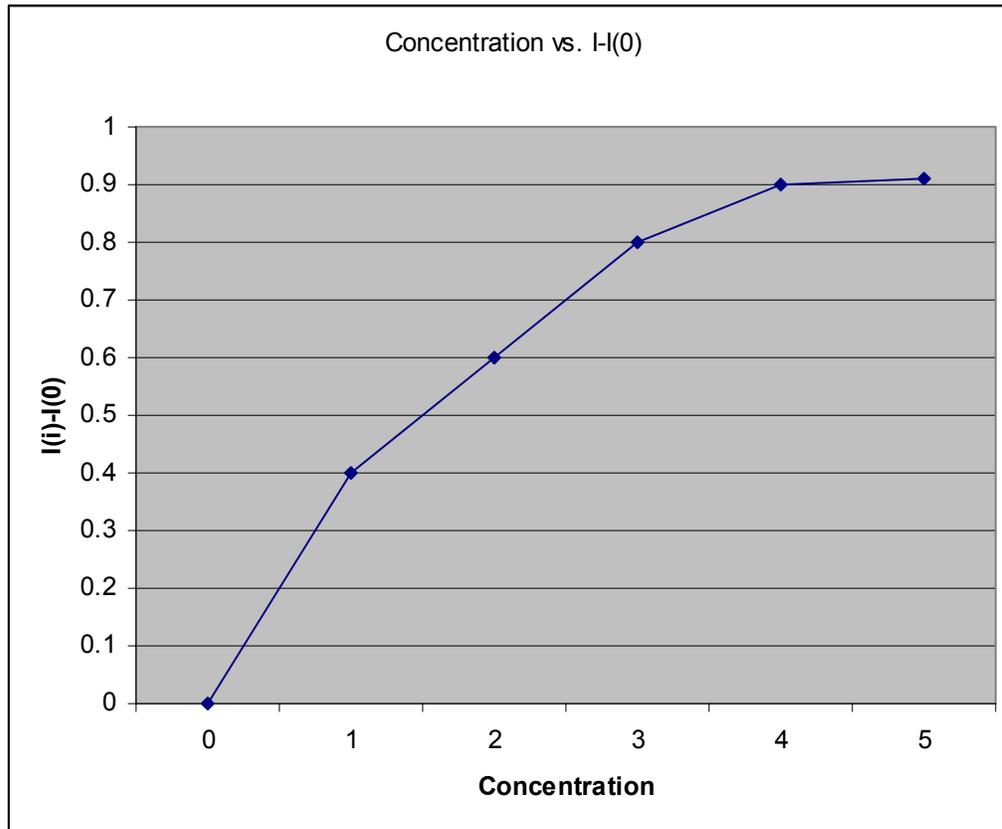
In the above example Δi approaches 0.9pA. Note that once found, this measure of the current drop can be used for all concentrations, but should be performed again for each different voltages applied (that is to say one should find Δi for each different voltage used.) Note that if the change in current does not approach a value, you may need to extend the graph with more concentration measurements. Alternatively, the system you are analyzing may not be a two-state system, in which case the above graph would not appear as a smooth transition and noise analysis may not be applicable.

Finally, the values of τ_b at a given concentration may be plotted against Voltage. The curves attained for different concentrations should be match.

Procedure for finding rate constants: The following procedure can be used to find rate constants 1 $\underset{k_2}{\overset{k_1}{\rightleftharpoons}}$ 2.

Assumptions: The system must be a two-state process. It must be a Markov process. The noise of the system must be Gaussian. The sampling rate interval, Δt, must be greater than $k = k_1 + k_2$, such that $k\Delta t \geq 10$.

Data Acquisition:
Data Acquisition should be performed using a Butterworth filter in conjunction with other standard single channel electrophysiology equipment (see diagram below)
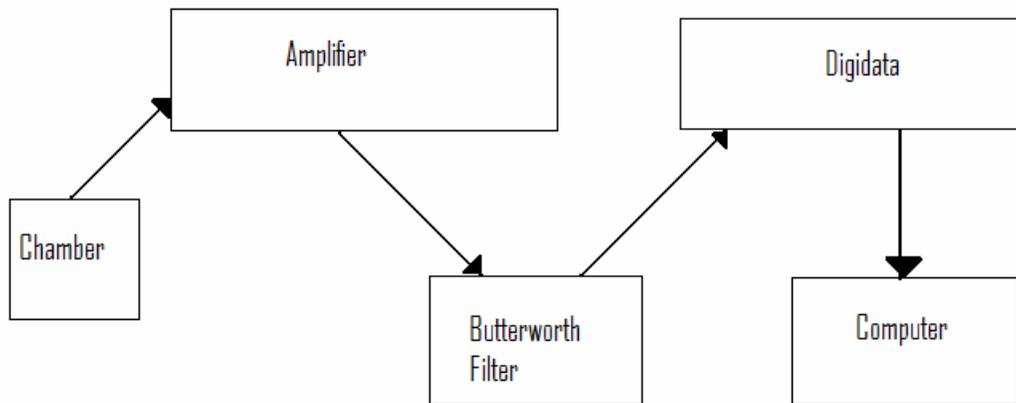
If there are previous estimates of the rate constant $k = k_1 + k_2$, make sure to set the frequency of the Butterworth filter is set $\leq \dfrac{(k_1 + k_2)}{10}$. The frequency of the Butterworth should be made as high as possible without violating this equation. In most cases it will be set to the maximum of 50kHz. The Digidata frequency should be set to at least 2.5 times the Butterworth frequency. For example, if the Butterworth is set to 50kHz, a frequency of 200kHz is appropriate for the Digidata.
Current traces should be acquired at a given concentration and voltage (~200mV.) A base current trace should be made as a reference.

Parameters:
I_1 is the current when in state 1.
I_2 is the current when in state 2.
ΔI is the difference in current between states 1 and 2. (This can be estimated as before)
I_{eq} is estimated as the average current at equilibrium.
σ_I^2 is estimated as the variance in current at equilibrium.

Δt is the sampling rate interval.
D is the amplitude of the (Gaussian) noise.

I_1 can be found by taking the average of the current trace with no analytes (see pags 2-3). ΔI is the change in current when the analyte is in the channel. This can be found by saturating the pore with increasing analyte concentration. The procedure is the same as that followed on pages 4-5. Increase the concentration of analytes until the current at a given voltage approaches a constant value. I_2 can be found by the formula $I_1 - \Delta I$.

Finding I_{eq} and σ_I^2 for a given analyte concentration and voltage: Select the desired current trace in an open window, then select the region you wish to analyze with cursors then go to Analyze→Histogram. Select the appropriate cursors. Press OK. You may have to adjust the bin number if the resulting histogram does not look Gaussian. When the Histogram window opens, go to Analyze→Fit and select "Gaussian" under predefined function. This should result in a window similar to the one displayed below. I_{eq} is circled in red and σ_I in blue (be sure to square this quantity.)
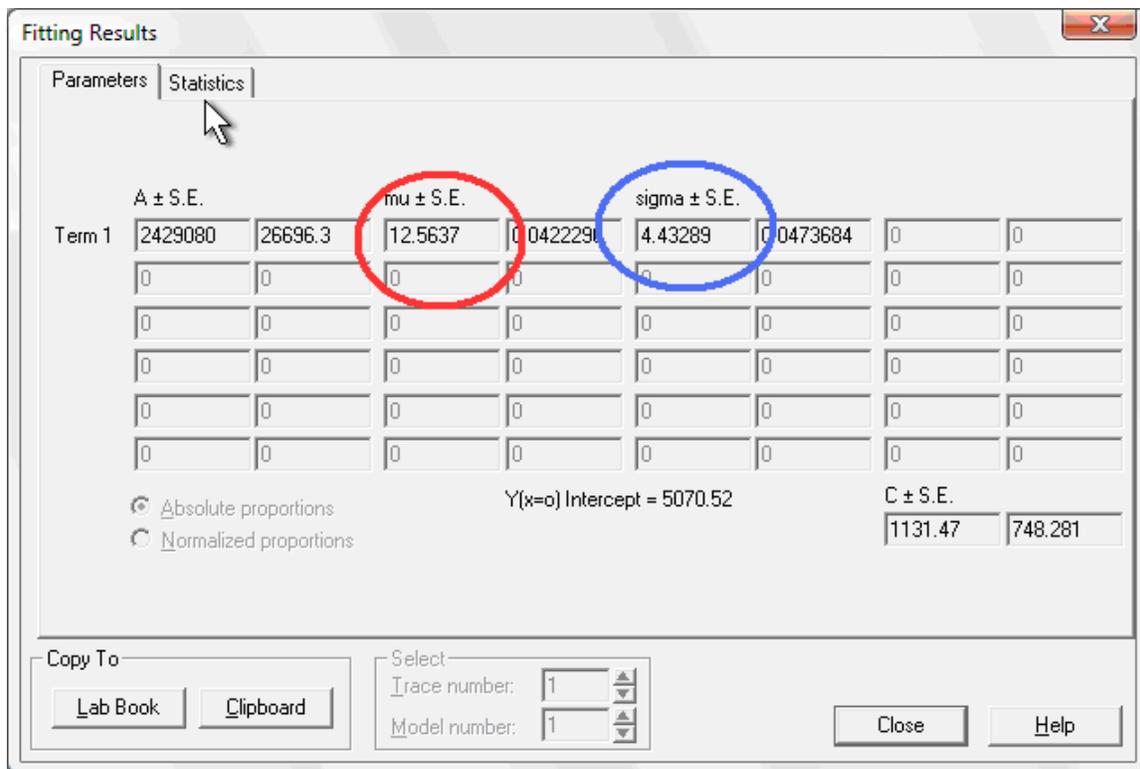
D can be measured directly from the current trace using the measure bars as the distance from the top to the bottom of the noise.

Using these data,

$$k_1 = \left(1 - \frac{I_{eq} - I_2}{\Delta I}\right) \frac{2(I_1 - I_{eq})(I_2 - I_{eq})}{\sigma^2(\Delta t)\Delta t - D}$$

$$k_2 = \left(\frac{I_{eq} - I_2}{\Delta I}\right) \frac{2(I_1 - I_{eq})(I_2 - I_{eq})}{\sigma^2(\Delta t)\Delta t - D}$$

References:

Bezrukov, S. M., O. V. Krasilnikov, L. N. Yuldasheva, A. M. Berezhkovskii, and C. G. Rodrigues. 2004. Field-Dependent Effect of Crown Ether (18-Crown-6) on Ionic Conductance of α-Hemolysin Channels. *Biophysical Journal*. 87: 3162-3171

Boguna, M., L. Kullman, S. M. Bezrukov, A. M. Berezhkovskii, and G. H. Weiss. 2001. Rate constants from uncorrelated single-molecule data. *J. Phys. Chem. 105:6246-6250.*

Machlup, S. 1954. Noise in semiconductors: spectrum of a two-parameter random signal. *J. Appl. Phys.* 25:341-343.