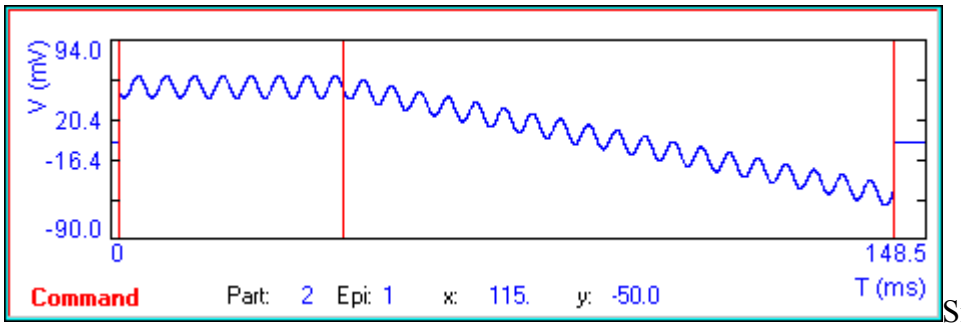
Technical Note 1

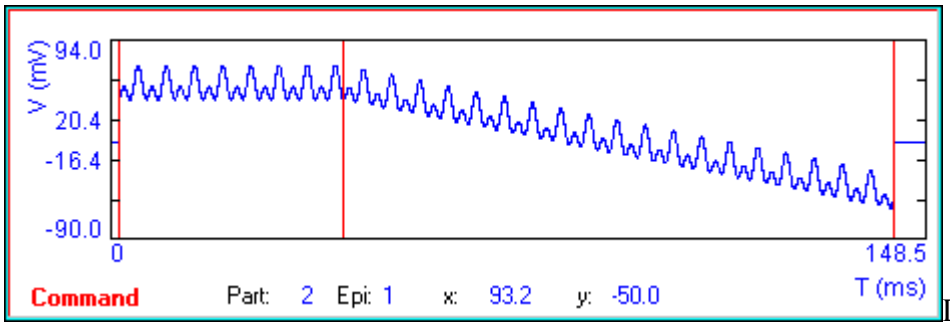
SciSoft Company (www.SciSoftCo.com)

Detrending of dual and single-sine data in jClamp -- detrending now exposed in jClamp

Cell membrane capacitance can be measured with the 2-sine or phase track technique in jClamp. Either a 2-sine or single sine stimulus can be superimposed on any waveform created in the Command Utility.

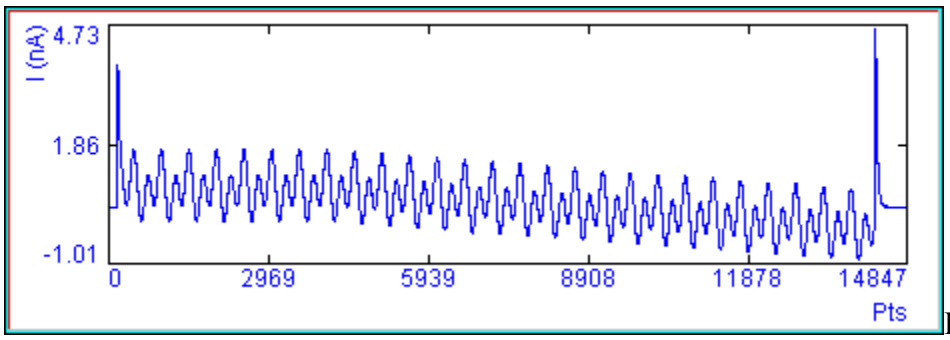


Single Sine.

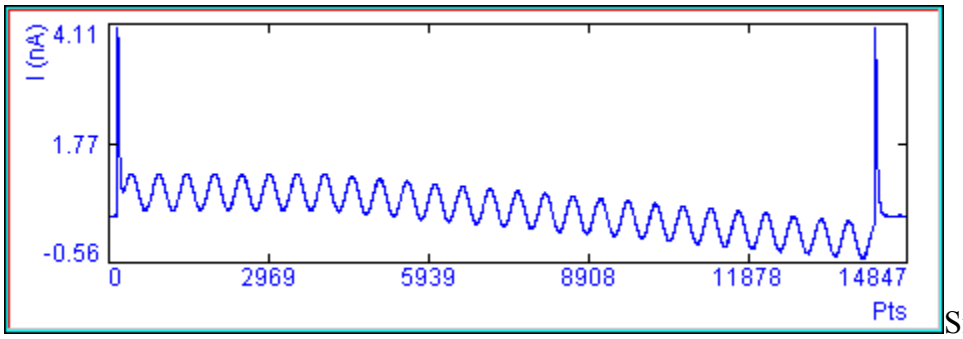


Dual Sine.

The response from a simple cell model is shown below.



Dual Sine.



Single Sine.

The response from dual sine stimulation is analyzed with FFT to obtain the real and imaginary components at each stimulus frequency. These data are used to determine the *parameters* of a simple model of the recorded cell – a **series resistance** in series with a parallel combination of a **membrane resistance** and **membrane capacitance**. The equations are from Pusch and Neher, 1988. I outlined the application of their equations to the novel 2-sine technique in one of my grant proposals in 1992, which is photocopied in the appendix below, along with the final progress report of the grant. It makes for interesting reading!

Single sine stimulation can also be used to measure membrane capacitance with the same cell model.

See jClamp's help file for details.

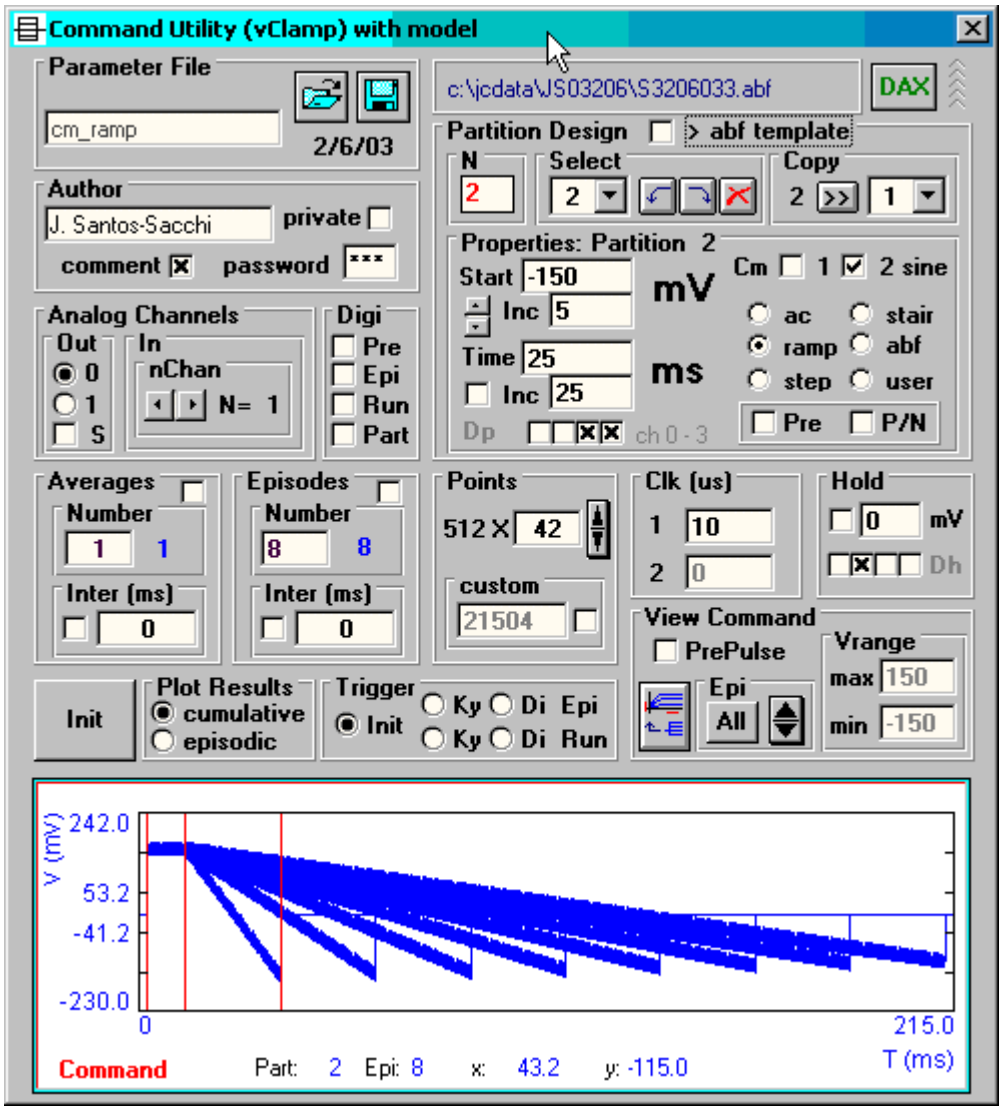
Each technique requires that you obtain a calibration file that is used to correct for the frequency response of the patch clamp system (electrode, amplifier, filters). Correcting for the system response is important since the *parameter* solutions are model dependent and assume that the system response is flat. It is the job of the calibration file to flatten the unflat system response. It is important for correct measurement that the electrode's stray capacitance (which is not included in the model) is balanced out during pipette-membrane seal formation.

Another important step to take for proper parameter estimation is detrending of the data. FFT measures of selected frequencies (in our case the frequencies of the stimulating sine waves) are best evaluated if other slow time dependent changes in the response are removed. In jClamp, if you superimpose the stimulating sine waves on a time varying stimulus, e.g. a ramp or slow sine wave, then it is best to remove this slowly changing response from the data prior to FFTing. I use a linear approach to detrending, but it is on a very small (relative) time scale so it avoids problems with whole dataset nonlinearity.

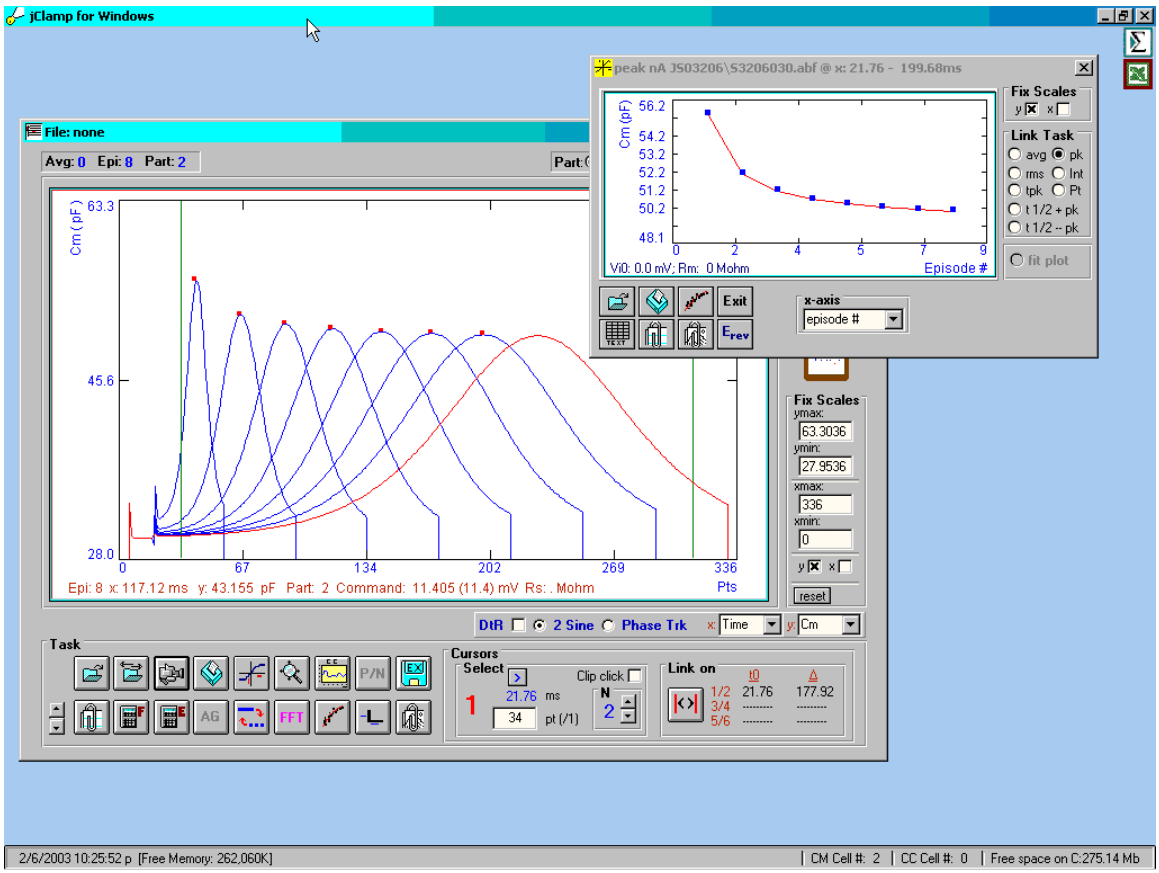
An example: Given a 10us clock and a 128 ms ramp with 2 sine stimuli superimposed at a resolution of 1.28 ms (these are all set in the command utility), there are 100 repetitions of a *unit* stimulus consisting of one period of the low frequency (f1) component and 2

periods of the high frequency (f2) component (the ratio of f2 to f1 is 2). I detrend on the scale of the *unit* stimulus, which for the whole stimulus (100 times larger) proves to be much better than full linear detrending, and I think on par with a nonlinear (spline) approach. The success of this "piece-wise linear" approach is shown below.

Here I have used the math model in jClamp to generate nonlinear responses owing to a voltage dependent capacitance. A difference equation technique is used in the model to obtain solutions. The nonlinear capacitance derives from the first derivative (re: voltage) of a simple two-state Boltzmann charge transfer. Below I stimulate with ramps (with the two sine technique) of differing rates using jClamp's incrementing time feature.

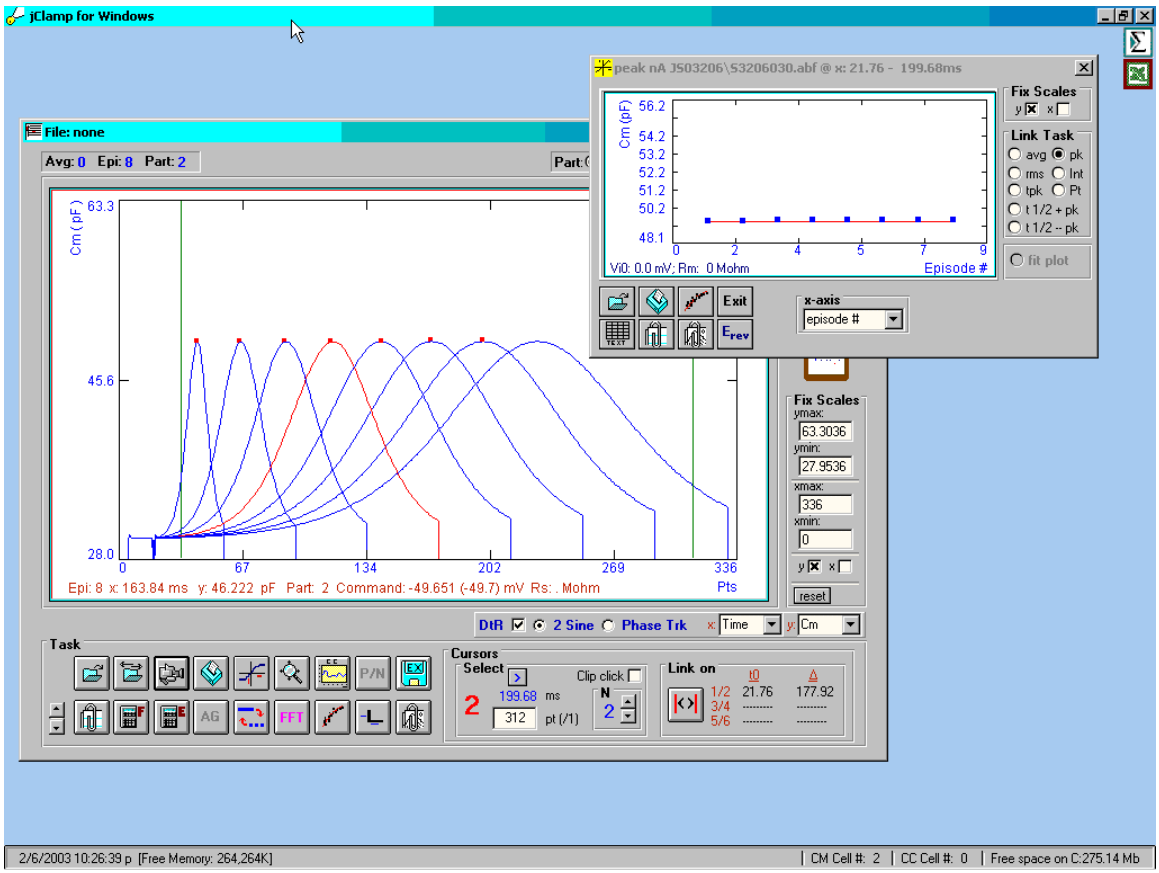


Below is the capacitance plot derived from the incrementing ramp stimulations.



In the analysis window I plot all episodes. It is clear that the magnitude of the peaks of capacitance depend on ramp rate, the faster the ramp rate, the larger the peak, even though the model parameters are the same for each ramp data collection. The I-V plot window clearly shows the relationship.

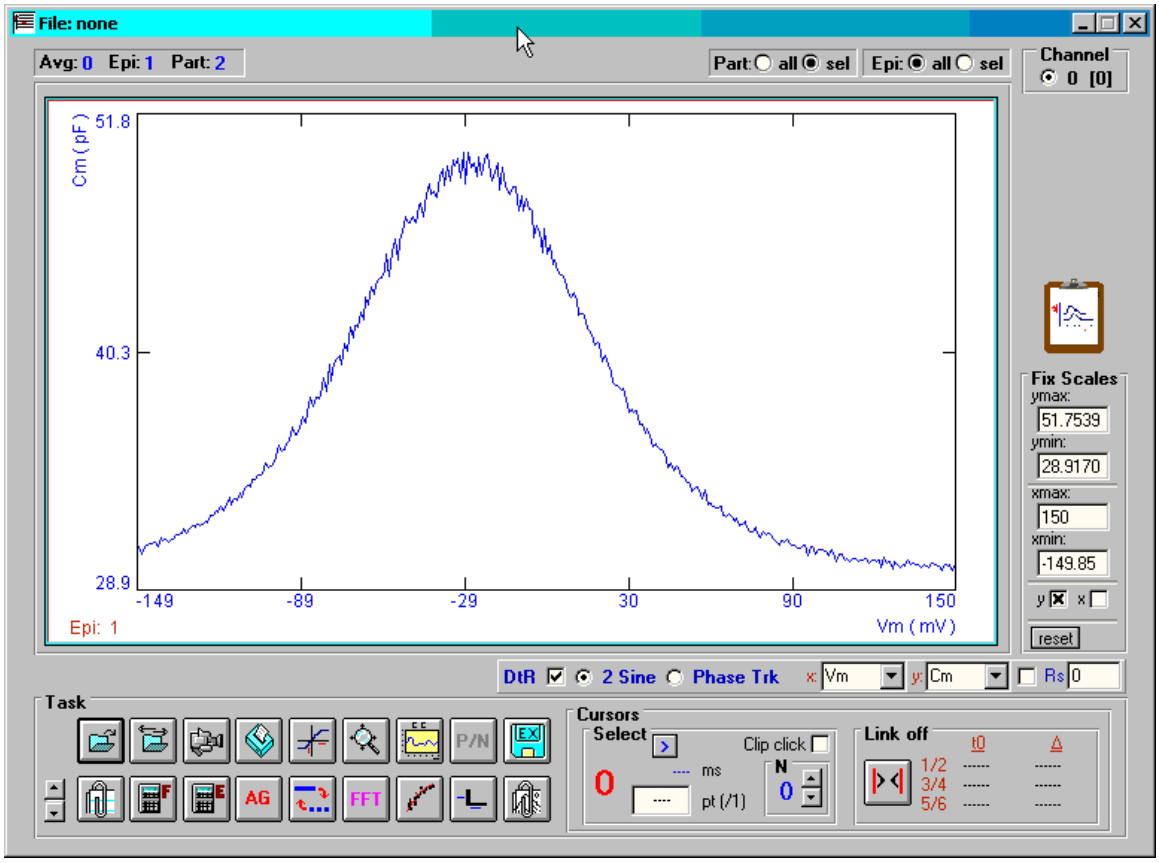
Several years ago I implemented detrending in jClamp because of this potential problem. If you use only step stimuli then these is no rate problem (except at onset!). Below is the response when the detrending feature of jClamp is enabled.



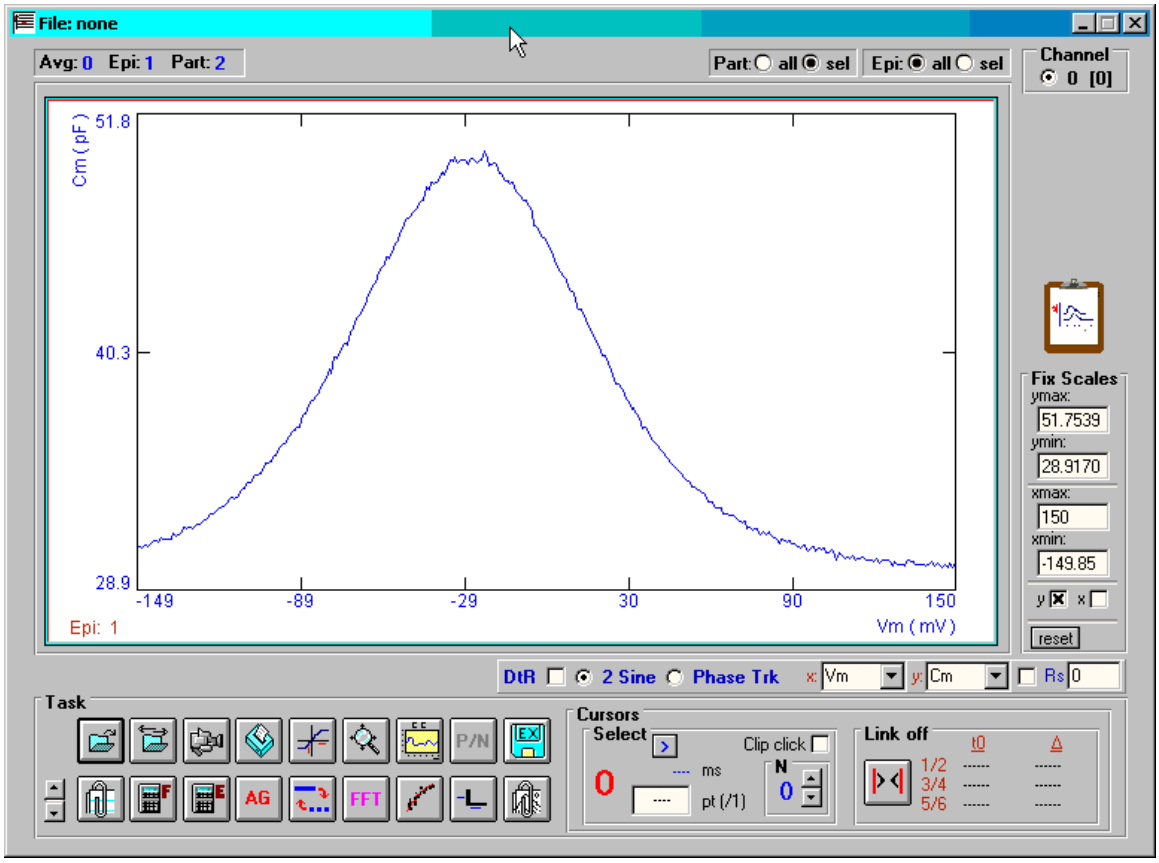
The response is in accord with the set model parameters. I should note that the generation of currents by the model is independent of the analysis procedure, so it is unlikely that the effect of ramp rate is due to model evaluation with the difference equation technique. Furthermore, the clock was sufficiently fast and the same for all ramps -- 10 us.

I have looked at real data and the effects of ramp speed are not so obvious as in model data, but to play it safe, I implemented detrending several years ago in jClamp. Recently, (jClamp V. 10.5) I have exposed detrending to the user, so that you may enable or disable it. It is probably a good approach for analysis of data obtained with stimuli of rapid rates, but I have found that it may introduce some noise into the measurements.

Below I have added noise to the current response prior to analysis. If detrend is used then the noise depends on the magnitude of the capacitance, however if detrend is off then the noise is small and uniform in the face of capacitance increase.



With detrending (**DtR** check box checked).



No detrending (**DtR** checkbox unchecked).

You can now decide whether to use detrending. If the underlying stimulus rate is fast and a small noise increase is not crucial then use detrending, else disable detrending.

AA

Form Approved Through 6/30/
OMB No. 0925-00

DEPARTMENT OF HEALTH AND HUMAN SERVICES
PUBLIC HEALTH SERVICE

GRANT APPLICATION

Follow instructions carefully. Type in the unshaded areas only.
Type density must be 10 c.p.i.

LEAVE BLANK FOR PHS USE ONLY.		
Type	Activity	Number
Review Group		Formerly
Council/Board (Month, Year)		Date Received

1. TITLE OF PROJECT (Do not exceed 56 typewriter spaces.)
In Vivo Analysis of Outer Hair Cell Motility

2a. RESPONSE TO SPECIFIC REQUEST FOR APPLICATIONS OR PROGRAM ANNOUNCEMENT ☐ NO ☐ YES (If "YES," state number and title)
Number: Title:

2b. TYPE OF GRANT PROGRAM: RO1

3. PRINCIPAL INVESTIGATOR/PROGRAM DIRECTOR

3a. NAME (Last, first, middle)
Santos-Sacchi, Joseph

3b. DEGREE(S)
A.B. MPhil Ph.D.

3c. SOCIAL SECURITY NO.

3d. POSITION TITLE
Associate Professor

3e. MAILING ADDRESS (Street, city, state, zip code)
Dep't of Surgery/Otolaryngology
Yale Univ School of Medicine
333 Cedar Street
New Haven, CT 06510

3f. DEPARTMENT, SERVICE, LABORATORY, OR EQUIVALENT
Surgery

3g. MAJOR SUBDIVISION
School of Medicine

3h. TELEPHONE AND FAX (Area code, number and extension)
TEL: 203 785-7566
FAX: 203 737-2245

BITNET/INTERNET ADDRESS: SANTOS@YALEMED

4. HUMAN SUBJECTS — 4a. X NO ☐ YES — If "Yes," exemption no. or IRB approval date — 4b. Assurance of compliance no.: M1452

5. VERTEBRATE ANIMALS — 5a. ☐ NO X YES — If "Yes," IACUC approval date: Pending — 5b. Animal welfare assurance no.: A3230-01

6. DATES OF ENTIRE PROPOSED PROJECT PERIOD		7. COSTS REQUESTED FOR INITIAL BUDGET PERIOD		8. COSTS REQUESTED FOR ENTIRE PROPOSED PROJECT PERIOD	
From (MMDDYY)	Through (MMDDYY)	7a. Direct Costs ($)	7b. Total Costs ($)	8a. Direct Costs ($)	8b. Total Costs ($)
070193	063098	102,377	157,975	501,142	808,750

9. PERFORMANCE SITES (Organizations and addresses)
Yale Univ School of Medicine
333 Cedar Street
New Haven, CT 06510

10. INVENTIONS AND PATENTS (Competing continuation application only)
☐ NO ☐ YES If "YES," ☐ Previously reported ☐ Not previously reported

11. NAME OF APPLICANT ORGANIZATION
Yale University
ADDRESS: 155 Whitney Avenue
New Haven, CT 06520

12. TYPE OF ORGANIZATION
☐ Public: Specify ☐ Federal ☐ State ☐ Local
☒ Private Nonprofit
☐ Forprofit (General) ☐ Forprofit (Small Business)

13. ENTITY IDENTIFICATION NUMBER: 060646973
Congressional District: 3rd

14. BIOMEDICAL RESEARCH SUPPORT GRANT CREDIT
Code: 01 Identification: School of Medicine

15. NAME OF ADMINISTRATIVE OFFICIAL TO BE NOTIFIED IF AWARD IS MADE
H.G. Aaslestad, Ph.D.
TELEPHONE: 203 785-4689
FAX: 203 785-4159
TITLE: Assoc. Dean for Research Affairs
ADDRESS: Yale University
School of Medicine
333 Cedar Street
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BITNET/INTERNET ADDRESS

16. NAME OF OFFICIAL SIGNING FOR APPLICANT ORGANIZATION
H.G. Aaslestad/V.M. Lingis
TELEPHONE: 203 785-4689
FAX: 203 785-4159
TITLE: Director/Associate Director
ADDRESS: Grant & Contract Administration
Yale Univ School of Medicine
333 Cedar Street
New Haven, CT 06510
BITNET/INTERNET ADDRESS

17. PRINCIPAL INVESTIGATOR/PROGRAM DIRECTOR ASSURANCE: I agree to accept responsibility for the scientific conduct of the project and to provide the required progress reports if a grant is awarded as a result of this application. Willful provision of false information is a criminal offense (U.S. Code, Title 18, Section 1001). I am aware that any false, fictitious, or fraudulent statement may, in addition to other remedies available to the Government, subject me to civil penalties under the Program Fraud Civil Remedies Act of 1986 (45 CFR 79).

SIGNATURE OF PERSON NAMED IN 3a. (In ink. "Per" signature not acceptable.) J. Santos Sacchi — DATE: 9/23/92

18. CERTIFICATION AND ACCEPTANCE: I certify that the statements herein are true and complete to the best of my knowledge, and accept the obligation to comply with Public Health Service terms and conditions if a grant is awarded as the result of this application. A willfully false certification is a criminal offense (U.S. Code, Title 18, Section 1001). I am aware that any false, fictitious, or fraudulent statement may, in addition to other remedies available to the Government, subject me to civil penalties under the Program Fraud Civil Remedies Act of 1986 (45 CFR 79).

SIGNATURE OF PERSON NAMED IN 16. (In ink. "Per" signature not acceptable.) Verna M. Lingis — DATE: 9/28/92

a) Acoustical analysis

For this analysis, a swept frequency burst (chirp; for third turn: 100-3000Hz) or discrete tone bursts will be delivered into the sealed ear canal and the averaged receptor potential response will be analyzed by FFT. The cutoff frequency (f_c; as determined from 45 degree phase lag re: organ of Corti potentials [Dallos and Santos-Sacchi, 1983]) will be determined. The cutoff should correspond to the (RC) time constant of the membrane; however, the time constant will be influenced not only by any voltage dependent capacitance but also by changes in voltage dependent conductances activated by the auditory stimulus and biasing voltage. In order to reduce activation of voltage dependent conductances by sound, SPL levels will be kept as low as possible. The biasing currents, on the other hand, are bound to evoke drastic changes in resting conductance (Santos-Sacchi and Dilger, 1988), and these changes will be mirrored in cutoff frequencies. However, since the cutoff frequencies are thought to be principally determined by the RC combination of the cell membrane, it may be possible to take into account the induced changes in membrane resistance. To this end, small current pulses (50-100 pA; 100 msec) will be delivered during the bias steps in order to determine the new steady state membrane resistance. Once this is known, it is possible that the cutoff frequency (i.e., membrane time constant) can be utilized to determine the capacitance at that membrane potential.

b) Electrical analysis

In addition, to the acoustical determination of OHC membrane capacitance, an electrical technique can be employed. That is, using small signal AC analysis it may be possible to determine the characteristics of the system under study.

I have used wideband AC analysis to determine OHC voltage dependent capacitance under in vitro voltage clamp (Santos-Sacchi, 1991). For such experiments, an OHC is injected with a small AC signal, and the impedance of the system is evaluated based on a simple model of the electrode resistance (R_s; the electrode's capacitance is compensated electrically with the recording amplifier), in series with the parallel combination of the cell capacitance (C_m) and resistance (R_m). In the present experiment, a discrete frequency analysis will be attempted. Pusch and Neher, (1988) have used a single "tone" (sine wave, actually) technique to determine cell characteristics under whole cell voltage clamp. By injecting a sinusoidal voltage into a cell, and measuring the current response, they calculated from the system's impedance, the cell capacitance, resistance and electrode resistance.

$$R_s=(A-b) \;/\; (A^2 + B^2 - A*b)$$
$$R_m=1/b * ((A-b)^2 + B^2) \;/\; (A^2 + B^2 - A*b)$$
$$C_m=(1 \;/\; (\omega * B))) * ((A^2 + B^2 - A*b)^2 \;/\; ((A-b)^2 + B^2))$$

where,

$b=1/(R_s+R_m)$
$Y=1 / Zin$
$A=Re(Y)$
$B=Im(Y)$
$\omega=2 \pi f$

As can be seen, with the use of one "tone" (ω), it is necessary to obtain an independent estimate of the input resistance (b). However, the utilization of a "two tone" ($\omega 1$ and $\omega 2$) stimulus allows one to determine all parameters, given the signal and response. That is, b need not be determined independently, and can be solved for, since, for example, R_s is the same at any two frequencies. The evaluations should work under voltage or current clamp. It is important however, that the AC stimulus itself induce insignificant changes in membrane characteristics. Since the membrane is responsive to changes in membrane potential, but the cell will be under current clamp, it is important that injected AC currents not perturb the membrane potential too much. Since the effect of an injected current on membrane potential will depend mainly upon the membrane resistance (for low frequency stimulation, e.g., $\omega 1$ and $\omega 2 < 200$ Hz), the membrane resistance will be measured with small current steps at each "holding potential" as above, and the injected AC currents will be sized so as to induce voltage responses of less than a few millivolts -- small enough to evoke minimal changes in membrane properties. In addition to the discrete frequency analysis, a wideband signal analysis utilizing fits to the frequency dependent impedance will be attempted (Santos-Sacchi, 1991). The program MATLAB will be used for FFT based impedance analyses. The voltage vs. capacitance function will be fit with a Boltzmann function (Santos-Sacchi, 1991), and will be compared to the parameters of the Boltzmann fit to BM motility data. See Specific Aim 4 for potential problems with these measures which may arise due to gap junctional coupling.

3) Does transient hypoxia alter the voltage dependence of the motility and nonlinear capacitance functions?

We have preliminary evidence that V_h shifts with phosphorylation, indicating that modulation of ATP reserves may indirectly alter the voltage dependence of OHC motility (Huang and Santos-Sacchi, 1993). In addition, it is possible that intracellular pH can cause shifts as well (Ashmore, 1990). The effects of transient hypoxia will be evaluated to determine if V_h reversibly shifts during ischemia. The protocols of Specific Aims 1 and 2 will be followed, and the determination of V_h will be made before, during and after recovery from hypoxia induced by ventilator shut-off. It may be possible that the length of time required to obtain a complete data set will be too long for complete recovery to occur. The logistics of this experiment can only be worked out once data collection times have been evaluated. Nevertheless, it will be possible to obtain pre and post hypoxia results.

Joseph Santos-Sacchi
Yale University
NIH NIDCD R01 DC02003

In 1992, I submitted and subsequently received funding for NIH grant R01 DC02003. Originally submitted as a five-year project, it was funded for 3 years. The specific aims are listed below.

Specific Aims

Answers to the following questions may help to understand the significance of OHC motility in vivo.

1) Is it possible to measure the effects of the electrically evoked motility of a single OHC on basilar membrane motion?

2) What is the voltage dependence of OHC nonlinear capacitance in vivo?

3) Does transient hypoxia alter the voltage dependence of the in vivo motility and nonlinear capacitance functions?

4) Are OHCs coupled via gap junctions to supporting cells?

The first year of the grant was occupied mainly by purchasing and setting up the in vivo recording equipment – including an IAC sound booth. During that time Mammano and Ashmore (1993) published a manuscript in Nature which effectively answered my specific aim 1. That is, in an isolated cochlea prep (in vivo-like), electrical stimulation of OHCs caused a clear deformation of the cochlear partition.

The other specific aims had as their methodological basis a technique that I proposed to measure membrane capacitance. That is, using small signal AC analysis it may be possible to determine the characteristics of the system under study. I have used wideband AC analysis to determine OHC voltage dependent capacitance under in vitro voltage clamp (Santos-Sacchi, 1991). For such experiments, an OHC is injected with a small AC signal, and the impedance of the system is evaluated based on a simple model of the electrode resistance (Rs; the electrode's capacitance is compensated electrically with the recording amplifier), in series with the parallel combination of the cell capacitance (Cm) and resistance (Rm). In the grant proposal, a discrete frequency analysis was to be attempted. Pusch and Neher, (1988) used a single "tone" (sine wave, actually) technique to determine cell characteristics under whole cell voltage clamp. By injecting a sinusoidal voltage into a cell, and measuring the current response, they calculated from the system's impedance, the cell capacitance, resistance and electrode resistance.

$Rs=(A-b) / (A2 + B2 - A*b)$
$Rm=1/b * ((A-b)2 + B2) / (A2 + B2 - A*b)$
$Cm=(1 / \omega * B))) * ((A2 + B2 - A*b)2 / ((A-b)2 + B2))$

where,

$b=1/(Rs+Rm)$
$Y=1 / Zin$
$A=Re(Y)$
$B=Im(Y)$
$\omega=2\pi f$

As can be seen, with the use of one "tone" (ω), it is necessary to obtain an independent estimate of the input resistance (b). However, the utilization of a "two tone" $\omega 1$ and $\omega 2$) stimulus allows one to determine all parameters, given the signal and response. That is, b need not be determined independently, and can be solved for, since, for example, Rs is the same at any two frequencies. The evaluations should work under voltage or current clamp. It is important however, that the AC stimulus itself induce insignificant changes in membrane characteristics.

This novel two sine wave approach to measure series resistance and membrane capacitance was written up as a short manuscript and submitted to the Biophysical J., but was rejected because it was deemed too "textbookish".

Later that year Dr. Neher visited Yale and I explained during his public lecture how this technique would be better than the one he currently employed. I even gave him a couple of pages from my grant application detailing the technique. Anyway, several months later I read in the Biophysical J an article entitled "A Novel Method for Rapid Measurement of Membrane Resistance, capacitance, and access resistance" by Donnelly (1994). Dr. Donnelly is a Research Scientist here at Yale Medical School. Somehow he devised a two-sine wave method to do these measures! This is not the first time that my speaking in public about unpublished information has ultimately displeased me.

In any case, we were not successful in utilizing the technique in vivo to measure OHC nonlinear capacitance. Basically, the problem was the difficulty obtaining stable recordings from OHCs that were very infrequently obtained. Instead we resorted to proving the technique in vitro, culminating in a manuscript that evaluated the influence of prior voltage on the voltage dependence of OHC capacitance and gating currents (Santos-Sacchi et al., 1998). We finally published on the technique that we originally devised! This manuscript, although performed in vitro, bears on the question posed in Specific Aim 2. It illustrates that the OHC resting voltage (membrane potential) and all factors which play a role in its generation and maintenance (viz., the electroanatomy of the cochlear partition) will affect the voltage dependence of OHC motility.

Subsequently, we adopted an in vitro preparation that mimicked the in vivo preparation to some extent. We used isolated pieces of the cochlear partition to answer some

questions posed in Specific Aims 1, 2 and 4; namely, are OHCs electrically coupled to each other or supporting cells, and what influence might the microanatomy of the cochlear partition have on OHC motility.

We directly measured, using dual whole cell voltage clamp, interactions between adjacent OHCs and supporting cells. We found no evidence for gap junction mediated coupling in OHCs. However, we did demonstrate that OHCs are mechano-electrically coupled to each other via attachments through Deiters' cells. The mechanical coupling among OHCs can modify the voltage dependence of OHC motility, and may be the mechanism whereby sharp tuning on the basilar membrane is obtained. A manuscript detailing our finding is published (Zhao and Santos-Sacchi, 1999). Additionally, manuscripts reviewing cell coupling (electrical and mechanical) in the organ of Corti (Santos-Sacchi, 1999b), and the OHC (Santos-Sacchi, 1999a) have resulted.

We believe that through the help of this grant we have been able to uncover details of OHC function that may be important in vivo.

Resulting manuscripts:

Santos-Sacchi, J. On calculating series resistance and membrane capacitance. (submitted and rejected Biophysical J 1993)

Santos-Sacchi, J., Kakehata, S. and Takahashi, S. Effects of membrane potential on the voltage dependence of motility-related gating charge in outer hair cells of the guinea pig. J. Physiology (London) 510:225-235. 1998

Zhao HB, Santos-Sacchi J Auditory collusion and a coupled couple of outer hair cells. Nature 399: (6734) 359-362 MAY 27 1999

Santos-Sacchi, J. The magnificent outer hair cell of Corti's organ. Otol Jpn 9, 111-115, 1999a

Santos-Sacchi, J. Cell coupling in Corti's organ. 1999b, In press, Brain Research

References:

Mammano, F. & Ashmore, J. F. Reverse transduction measured in the isolated cochlea by laser Michelson interferometry. *Nature* **365,** 838-841 (1993).

Donnelly, DF 1994. A Novel Method for Rapid Measurement of Membrane Resistance, capacitance, and access resistance Biophysical Journal 66: 873-877.

Santos-Sacchi J. Reversible inhibition of voltage-dependent outer hair cell motility and capacitance J Neurosci 11: (10) 3096-3110 1991