
Supplementary information

Regulation of the mammalian-brain V-ATPase through ultraslow mode-switching

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Regulation of the mammalian-brain V-ATPase via ultra-slow mode-switching

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Supplementary discussion

Size calibration

We used previously described protocols in order to convert from fluorescence intensities to size distributions^{1,2}. Briefly, a series of widefield images of the immobilized vesicles were acquired and averaged before the start of activity measurements. The absolute fluorescence intensity of each vesicle scales with the number of pHrodo-DOPE molecules which in turn scales with the surface area of the bilayer and therefore the radius of the vesicle according to the equation:

$$R = k \cdot \sqrt{I_{\text{pHrodo-DOPE}}},$$

where R is the radius of the observed vesicle, I is the fluorescence intensity observed. k is a conversion factor that is correlating the mean radii as determined by distributions of vesicle sizes obtained by cryoEM (Fig. S2b) to mean fluorescence intensities obtained from the widefield images (Fig. S2c). k is calculated by dividing the Gaussian mean of the radii by the square root of the Gaussian mean of the widefield intensity, such that $k = \bar{R} / \sqrt{\bar{I}_{\text{pHrodo-DOPE}}}$.

Stochastic model description

In the following the ‘signal’ will refer to the increased intensities of light in the experiment due to the V-ATPase being active. The signal depends on change-point times $\mathbf{T} = \{T_1, T_2, \dots, T_n\}$ which are the times of mode-switching events at which the V-ATPase switches from an on-cycle state to an off-cycle state or visa versa. These states alternate, such that T_i represents the point at which the proton pump switches to a proton-pumping mode if i is odd or an inactive mode if i is even.

In the proton-pumping mode, protons are pumped inside the vesicle, and so the fluorescence increases. This is modelled as an exponential increase up to a dynamic equilibrium of the system at which point the influx of protons due to the V-ATPase is equal to the passive efflux through the membrane. This equilibrium level we call α and the exponential rate as β . Then when the V-ATPase switches to an inactive-mode we model the fluorescence decreasing exponentially back to the baseline. The rate of this exponential decay we call γ . These exponential increases and decays from a general starting point K , between the baseline and equilibrium level α , can then be represented as follows $\mu_1(t; K) = (K - \alpha) \exp(-\beta t) + \alpha$ and $\mu_2(t; K) = K \exp(-\gamma t)$. As the

signal is switching state with every mode-switching event the general formula/model for the signal is

$$\mu(t) = \begin{cases} \mu_2(t; 0) & \text{if } 0 \leq t < T_1 \\ \mu_1(t - T_1; \mu(T_1)) & \text{if } T_1 \leq t < T_2 \\ \mu_2(t - T_2; \mu(T_2)) & \text{if } T_2 \leq t < T_3 \\ \mu_1(t - T_3; \mu(T_3)) & \text{if } T_3 \leq t < T_4 \\ \vdots & \vdots \end{cases}$$

noting that $\mu_2(t; 0) = 0$.

In this setting, we allow the exponential rates to be different from those seen previously (but fixed between mode-switching events), so for example we will denote β_1 and γ_1 to be the exponential rates of the first proton-pumping event and first inactive event respectively. However, we will assume the equilibrium level for the cell α to be the same for any point in time. For ease of notation, we will group these parameters together as $\boldsymbol{\nu} = \{\alpha, \beta_1, \gamma_1, \dots, \beta_n, \gamma_n\}$. Letting the time between mode-switching events be defined as $\boldsymbol{\theta} = \{\theta_1, \theta_2, \dots, \theta_n\} = \{T_1, T_2 - T_1, \dots, T_n - T_{n-1}\}$, and note that the signal's dependence on $\boldsymbol{T}$ can be replaced by $\boldsymbol{\theta}$ as one set can be constructed from the other. We shall write the general formula for the signal as $\mu(t, \boldsymbol{\theta}, \boldsymbol{\nu})$.

Each signal depends uniquely on the equilibrium level for the vesicle, exponential rates in the model and also the times between mode-switching events which are not known a priori. These unknowns are modelled as random variables with given unbiased prior distributions. In this Bayesian setting, it is assumed that all the random variables are mutually independent and the distributions are given as follows; the time between change-points is exponentially distributed (highest entropy on the positive real numbers); the equilibrium level for the cell α is uniformly distributed (highest entropy over a given interval); the distribution for the exponential rates β and γ are a uniform distribution over an approximately even spread for the slopes of the exponential functions μ_i . Using unbiased prior distributions ensures that these priors have the least impact on the a posteriori estimates that are calculated (see next section).

Stochastic filtering

This section looks to give a brief overview of how techniques from Bayesian stochastic filtering are used in the context of this paper. For an introduction to stochastic filtering techniques see ³. Bayesian stochastic filtering is a method of filtering out the unwanted noise leaving an estimate of

the signal (given the model described above). The method takes the a priori opinion of the signal's change-points and parameters in the form of the unbiased prior distributions and provides an updated opinion given the data, known as the a posteriori (joint) distribution. This is then used to find the maximum a posterior probability (MAP) estimate of the signal. In calculating the a posteriori joint distribution further important qualities can be derived including the marginal posterior distribution of each individual change-point and parameter, along with useful information like the probability that there are x number of change-points in the observed data.

The observed data from the experiments are the unobserved signal of increased intensities of light in the experiment due to the proton pump being active plus noise of random variations due to various different sources (e.g. camera noise, biological noise). Before initiation of activity by addition of ATP, single vesicle data contains a sample from which the variance of the noise and the base line around which the samples (on average) fluctuate is calculated. Without loss of generality, we consider the standardized case where the variance equals 1 and the baseline is at 0.

As the entire trajectory of the observed process in continuous time is not available, we operate with observations that are sampled at times $t = 1, 2, \dots, n$ (again for notational simplicity). Thus the observed data are $\mathbf{X} = (X_1, X_2, \dots, X_n)$. As there is a finite set of observed data there is some probability only the first few (or indeed none) of the change-points considered are in this set. Hence the method is robust so long as there are less change-points present in the data than the number of change-points allowed in the model.

The likelihood function L defined below can be seen as an approximation of the continuous time likelihood function using the discrete observed data

$$L(\boldsymbol{\theta}, \boldsymbol{\nu} | \mathbf{X}) = \prod_{t=1}^n \exp(\mu(t, \boldsymbol{\theta}, \boldsymbol{\nu}) X_t + 0.5 \mu(t, \boldsymbol{\theta}, \boldsymbol{\nu})^2).$$

Equivalently, this can also be derived by using a discrete time model for the signal. Given the joint probability density function $f(s)$ of the mode-switching events $\boldsymbol{\theta}$ and the joint probability density function $g(\boldsymbol{\nu})$ of the parameters $\boldsymbol{\nu}$ (see the previous section), the unnormalized a posteriori joint distribution for estimating $\boldsymbol{\theta}, \boldsymbol{\nu}$ can be calculated as follows $L(s, \boldsymbol{\nu} | \mathbf{X}) f(s) g(\boldsymbol{\nu})$. Then the MAP estimate $\hat{\boldsymbol{\theta}}, \hat{\boldsymbol{\nu}}$ of the mode-switching events and parameters are given by the values that maximize

the unnormalized a posteriori joint distribution. Finally, these estimates give the MAP estimate of the signal to be $\mu(t, \hat{\theta}, \hat{\nu})$.

Using the normalized a posteriori distribution and integrating across all but one variable gives its marginal distributions (for example the a posteriori distribution of the first change-point). Given observed data $\mathbf{X}$, we can then find the probability that the first mode-switch event is present in this dataset.

Ideally the range of the distributions for α , β and γ would be as large as possible. However, due to numerical constraints, for the application of the method of Bayesian stochastic filtering on the real data we used the following constants to calculate the MAP estimates: The parameter of the exponential distributions of the mode-switching events is $10^{(-5)}$, α is in the range $\max(\mathbf{X}) - 2$ to $\max(\mathbf{X}) + 4$, and β 's and γ 's are in the range 0.01 to 3.93.

Sliding method

It can be seen that increasing the possible number of change-points in the problem increases the dimension of the joint distribution and so the numerical complexity of producing the MAP estimates. It is not known a priori how many mode-switch events will be present in a sample of observed data, so one option would be just to choose a number of mode-switch events that is large enough that the Bayesian stochastic filtering returns an estimate in which not all of the change-points looked for are observed in the data. However, even for a relatively small number of change-points (≥ 5) the numerical calculations can become highly time consuming, even with very efficient C++ code (C++11).

As MAP estimates were needed to be produced for a large number of samples, a novel sliding method of Bayesian stochastic filtering was developed which aimed to first segment the data into regions where there is only one mode-switch event and then produce the MAP estimate for these segments individually. This also involved updating our distribution of the vesicle equilibrium level α . This greatly reduced the complexity of required in the Bayesian stochastic filtering and made the method tractable for these large sets of data (see Table S1 for a description of the code).

Notes: 1) The Bayesian event detection algorithm described above is novel and unrelated to the Chung-Kennedy filter used here only as an aid for visualizing single molecule-traces. 2) It is exclusively used on raw data as it relies on the true noise of the system. 3) It was used not to

perform any sort of smoothing to the data but rather only to identify mode-switching events by analyzing the real/experimental noise.

Calculation of proton-pumping mode probabilities

The probability that the V-ATPase is found in a proton-pumping mode was calculated using $P_{pumping} = \sum t_{on} / (\sum t_{on} + \sum t_{off})$, except for Fig. 4d, where the probability as a function of ATP was estimated as $P_{pumping} = \sum t_{on} / t_{ATP}$, where t_{ATP} is the total time of the recording post ATP injection.

Physical model

We utilized the non-equilibrium model we developed in previous work to extract the pumping rates and permeabilities from single-molecule data^{2,4,5}. The acidification kinetics of single SV_h by the V-ATPase described by the following system of equations:

$$\frac{dpH_i}{dt} = -\frac{1}{\beta(pH_i)} \frac{d[H^+]_T}{dt}$$

$$\frac{dH_i^+}{dt} = I_p - (P_{leak}S + P_{V-ATPase}) \frac{U([H^+]_i - [H^+]_o e^{-U})}{1 - e^{-U}}$$

$$[H^+]_i = 10^{-pH}$$

$$\frac{dCl_i^-}{dt} = P_{Cl}S \frac{U([Cl^-]_i - [Cl^-]_o e^U)}{1 - e^U}$$

$$U = \frac{\Psi}{k_B T} = \frac{1}{k_B T} \frac{FV}{C_o S} \left(\left(-[Cl^-]_i + \int_{pH_o}^{pH_i} \beta(pH) dpH \right) - B \right).$$

Quantities in brackets correspond to concentrations of ionic species. Subscripts *i* and *o* correspond to intra- and extraluminal values respectively. β corresponds to the luminal buffering capacity of the system in which protons are buffered by both MOPS and pHrodo-DOPE molecules. I_p is the proton-pumping rate established by the V-ATPase. P_{leak} is the passive proton permeability of the SV_h membrane. S is the surface area and V is the volume of SV_h. S and V are experimentally determined following as mentioned above and in Fig. S2. $P_{V-ATPase}$ is the transprotein permeability. U is the reduced membrane potential. In our model, I_p , P_{leak} , and $P_{V-ATPase}$ are free parameters to be determined by the model. P_{Cl} is the chloride permeability of the system. For the modelling

performed in the present paper it was assumed that chloride can relatively freely permeate the membrane as a result of a plethora of mechanisms that transport chloride ions (VGluTs, Cl⁻/H⁺ antiport, passive permeation). Therefore, P_{Cl} is set to a large value. Ψ is the membrane potential. Single molecule traces fitted with the model are shown in E.D. Fig. 8. For more details regarding the model and the parameters involved we refer the reader to Supplementary Table 2 and ^{2,4,5}.

Calculation of the transition energy barrier heights for on-to-off and off-to-on cycle transitions.

Transition rates (main Fig. 3c, E.D. Fig. 6) were calculated using transition state theory, where the pumping and inactive modes are considered ground states separated by an activation barrier (the transition state). According to transition state theory the energy required to achieve the transition state (and cross the barrier) is:

$$\Delta G^\ddagger = -RT \ln \frac{h k_{i,j}}{k_B T}$$

where R is the gas constant, T is the absolute temperature, k_B is a Boltzmann's constant, h is Planck's constant, $k_{i,j}$ is the measured transition rate from pumping to inactivation or from inactivation to pumping states. Under the assumption that we have a two-mode model, proton-pumping and inactive mode rate constants can be calculated using equations

$$k_{on,off} = \frac{1}{\langle t_{on} \rangle}$$

$$k_{off,on} = \frac{1}{\langle t_{off} \rangle}$$

where $\langle t_{on} \rangle$ and $\langle t_{off} \rangle$ are the average dwell times in pumping and inactive modes for the V-ATPase.

Calculation of free energy difference between proton-pumping and inactive modes.

We calculated Gibbs free energy difference between proton pumping and inactive modes for different electrochemical conditions (E.D. Fig. 6) using equation,

$$\Delta G = -RT \ln \frac{P_{pumping}}{1 - P_{pumping}}$$

where $P_{pumping}$ and $1 - P_{pumping}$ represent the probabilities of the pump being in proton-pumping or inactive modes respectively.

Effects of membrane potential on mode-switching

The regulatory effects of $\Delta\Psi$ on τ_{on} and τ_{off} cancelled out each other, so that the overall probability of being in a proton-pumping mode remained independent of $\Delta\Psi$ within the experimental uncertainties, ($P_{on}^{+Cl} = 0.59 \pm 0.11$ and $P_{on}^{-Cl} = 0.54 \pm 0.16$, E.D. Fig. 6b). Notably, because the electrochemical gradient collapses back to zero during the periods of inactivity it cannot affect directly τ_{off} , thus the regulation of τ_{off} is likely the result of a molecular memory effect mediated via the proton pumping mode.

Concentration of active V-ATPases during microscopy measurements

After immobilization of LUVs and fusion with SVs, there are ~ 100 SV_h in a 0.0064 mm^2 FOV. Given that the area of the microfluidic chamber is 64.6 mm^2 and that there are 1.2 V-ATPases/SV_h, the total number of V-ATPases in the chamber is $1.2 \cdot 10^6$. Hence, given the chamber volume, $30 \text{ }\mu\text{l}$, the concentration of V-ATPases present during the course of an experiment is $\sim 70 \text{ fM}$.

Supplementary Methods

Image analysis

Data acquired from light microscopy experiments were analyzed using a custom software suite developed in the lab as well as custom scripts in MATLAB. A single particle localization algorithm identified single vesicles and their corresponding locations in the FOV. A drift correction algorithm was implemented whenever thermal fluctuations caused XY drifts during recordings⁶. Once image sequences have been stabilized and particles were identified⁷, we defined 5×5 pixel ROIs around each particle and got the integrated intensity readout by summation of intensity values of individual pixels. This is then applied throughout the image time series and therefore, single vesicle kinetics were produced. After each activity measurement, a stack of 20 images at saturating pH conditions was acquired to assist in the conversion of intensity to pH via calibration curves (for more details see section: Calibration of pH in vesicles). For pHrodo-PE the signal saturated at approximately pH 3 (E.D. Fig. 3).

Normalized intensity is defined as the raw intensity extracted divided by the mean intensity of the baseline, $I_{norm} = I_{raw}/I_{base}$. Standardized intensity is defined as the raw intensity reduced by the mean baseline intensity and divided by the standard deviation of the signal at the base line ($I_{std} =$

($I_{raw}-I_{base}$)/ σ_{base}). Normalized intensities were used as a typical representation of signals throughout this article. Standardized intensities were used by the stochastic filtering algorithm.

Active transport kinetics were initially identified via threshold filtering based on the signal-to-noise (SNR) of each kinetic trace. *SNR* is defined on an individual vesicle basis as the maximum normalized intensity reduced by one and divided by the normalized standard deviation of the baseline, $SNR = (I_{norm,max}-1)/\sigma_{norm,base}$. The selection of active transport kinetics was then manually classified based on the number of steps (intensity plateaus) identified (Fig. 1d, e). Only data with a single intensity step, corresponding to single transporter activity, was considered for single molecule data presented in the present article. Single vesicle activity traces that showed no stochastic behavior were not used in the single molecule data.

Dwell time analysis

A stochastic filtering algorithm (see Supplementary Discussion) was used to identify mode-switching events in single molecule data. In cases where there was overdetection of events, postprocess filtering algorithms were applied to clean up event data. Less than 10% of event data was subjected to postprocessing. A minor fraction of event detection data (less than 5%) was manually fine-tuned.

In order to calculate dwell times, we only considered single molecule data that showed at least one stochastic event. Proton-pumping dwell times (t_{on}) were calculated as the duration from the onset of acidification up until the point when the signal starts decreasing again which corresponds to the V-ATPase switching off (Fig. 2b). Inactive dwell times (t_{off}) are calculated as the duration from the start of signal decrease until the next on-cycle event (Fig. 2b). Dwell times of modes were only included in this analysis from the onset of the first proton-pumping event and were not interrupted by the ending of the recording. The probability that the V-ATPase is found in a pumping mode was calculated as the total time the V-ATPase spent in a pumping dwell versus the total time spent in both in pumping and inactive dwells post ATP injection ($P_{pumping} = \sum t_{on} / \sum (t_{on} + t_{off})$). Any event prior to the first pumping-dwell or interrupted by the end of the recording is rejected as the true duration of those events is unknown.

Calibration of pH in vesicles

In order to calibrate activity traces, a semi-local calibration methodology was followed (See also E.D. Fig. 3). Initially, we used DOPE-pHrodo labelled liposomes with the same lipid composition as mentioned previously (DOPC:DOPS:Chol:(18:1 Biotinyl Cap PE):DOPE-pHrodo 64.4:10:25:0.5:0.1) rehydrated in calibration buffer (150 mM K-Gluconate, 2 mM MOPS and 2 mM MgSO₄) and adjusted with Tris to reach a pH of 7.23. These liposomes were immobilized according to protocols mentioned above and they were incubated in calibration buffer which additionally included 60 nM valinomycin (val) and 5 μM Carbonyl cyanide m-chlorophenyl hydrazone (CCCP), to facilitate unconstrained influx of protons and efflux of potassium ions. Subsequently they were treated with calibration buffer at nine different pH values ranging from 7.23 to 3.19. For the calibration buffers, pH was adjusted with citric acid. For each pH measured, a stack of 20 images was recorded. Four replicates of this experimental process were performed. Using the custom software suite, we identified vesicles and extracted raw intensities for the different pH values recorded. For each vesicle the calibration data was fitted with the sigmoidal function:

$$I(pH) = I_{base} - \frac{I_{max}}{1 + e^{(pK_a - pH)/rate}} \quad (1)$$

I is the raw intensity recorded, I_{base} is the lower asymptote, I_{max} is the upper asymptote, pK_a is the acid dissociation constant and the $rate$ corresponds to the sharpness of the sigmoidal curve. Each fitted sigmoid then was normalized to the intensity corresponding to pH 7.1 as that corresponds to the pH of the baseline intensity of activity traces. We, then, extracted the mean values of the distributions of pK_a and rates. As the standard deviations of these parameter distributions are narrow the vesicle population displayed a well-defined and homogeneous behavior for these parameters. I_{base} and I_{max} , which define the dynamic range of the fluorophore, are more heterogeneous and thus are locally defined for each single SV_h after its activity has been acquired.

After recording single vesicle acidification kinetics in the activity buffer at pH 7.1, we incubate the SV_h for five minutes in the activity buffer, but at a signal saturating pH 2.85 in the presence of 5 μM CCCP and 30 mM choline chloride. Chloride is added to negate any membrane potential being established by proton influx. The absence of any other species of counterions (or neurotransmitters), such as potassium or sodium, ensures that secondary transporters, found on the

vesicle membrane, cannot transport substrates that could alter measuring conditions. Then, a sequence of twenty images is acquired under these conditions.

Calibration of activity data is done by first normalizing the raw traces. This is done by subtracting half of the baseline signal from the whole time series to correct for the fact that any signal variation reported due to activity of the V-ATPase is stemming from the pHrodo-PE lipids of the inner leaflet of the bilayer. Then, the trace is normalized to the new baseline intensity. Additionally, the raw intensity, I_{sat} , at saturating conditions (pH 2.85) is divided by two to compensate for contribution of pHrodo from both leaflets and then normalized to the baseline intensity. Solving equation (1) for pH we end with an expression of pH as a function of intensity and I_{base} and I_{max} are determined from the conditions pH = 7.1 when $I = I$ and pH = 2.85 when $I = I_{sat}$. It is assumed throughout this analysis that pHrodo-PE lipids are equally distributed between the two leaflets of the bilayer.

Proton leakage in hybrid vesicles and LUVs

We performed separate proton leakage experiments and also monitored leakage lifetimes during activity measurements in order to determine and deconvolve the leakage mechanisms. To determine passive leakage through the membrane, we immobilized LUVs on the glass surface and subsequently added SVs to initiate in situ fusion as described earlier. Subsequently, SV_h and LUVs are incubated for approximately 10 minutes in activity buffer (300 mM glycine, 2 mM MOPS and 2 mM MgSO₄) at pH 6.2 (pH is adjusted with citric acid). Then we injected activity buffer at pH 7.1. We record pH jumps from pH 6.2 to 7.1 and monitor the leakage kinetics by means of fluorescence signal reduction (Fig. S4). In order to distinguish between LUVs and SV_h, upon completion of the leakage measurement, we added 1 mM ATP and monitored activity thus allowing us to distinguish the two vesicle populations.

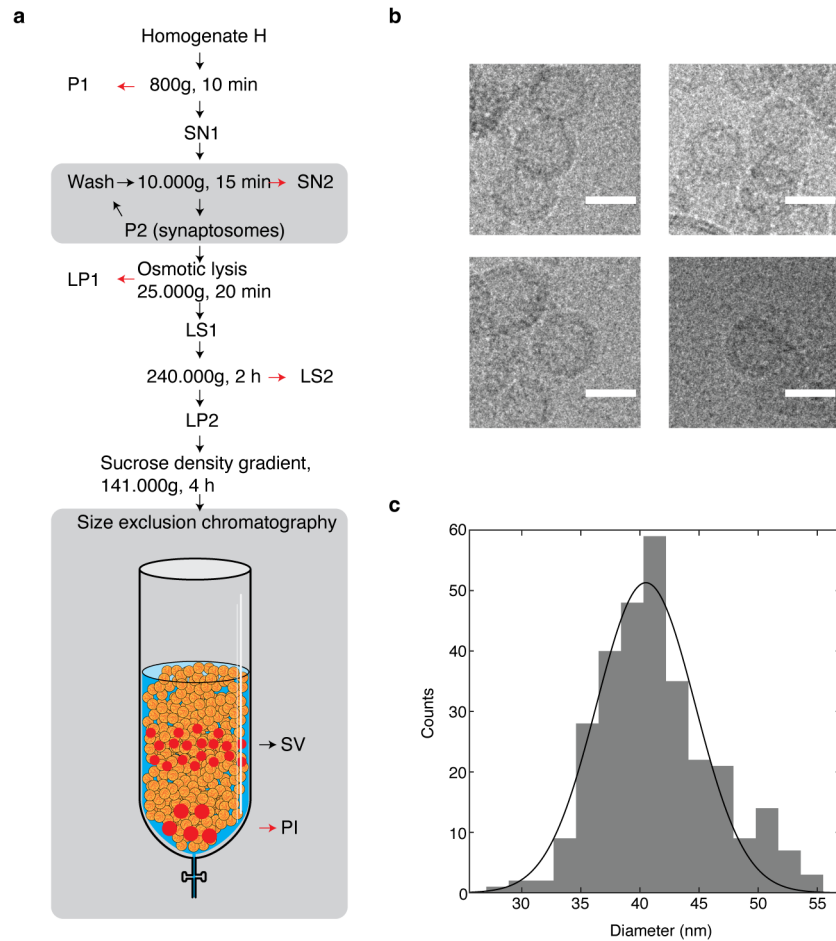
Once leakage data was collected for both LUVs and SV_h we fitted the single vesicle kinetics from the moment we did the buffer exchange with a double exponential function. The outer leaflet will respond fast to the buffer exchange, subject only to the flow rate induced by the peristaltic pump used to exchange media. Thus, the fast component of the fit corresponds to fluorescence stemming from the outer monolayer. Passive leakage through the vesicle membrane will be reported by the slow component of the double exponential. Leakage lifetimes of SV_h and LUVs were found to be in excellent agreement between them, strongly suggesting that no other protein on the membrane

of SV_h provides a pathway for protons to leak under our experimental conditions. Moreover, the fact that no stochastic jumps were observed during the pH jumps for either vesicle population indicates that any observed off-cycle modes are solely attributed to the V-ATPase. It is also noteworthy that no transprotein leak was observed for the V-ATPase in the absence of catalytic substrate or upon inhibition with bafilomycin.

Leakage lifetimes of SV_h during activity (in the presence of ATP) were estimated by fitting leakage rates with a mono exponential function when the V-ATPase switched to an inactive or leaky mode. Slow decay is attributed to membrane leakage while the fast decay stems from a transprotein leakage pathway through the V-ATPase (Fig. 2e). Due to the transprotein leak being 10 times faster than passive leakage through the membrane, a mono exponential function is sufficient to describe fast efflux kinetics. Lastly, single molecule leakage rates upon addition of 200 nm of bafilomycin were fit in a similar manner (Fig. 2e).

References

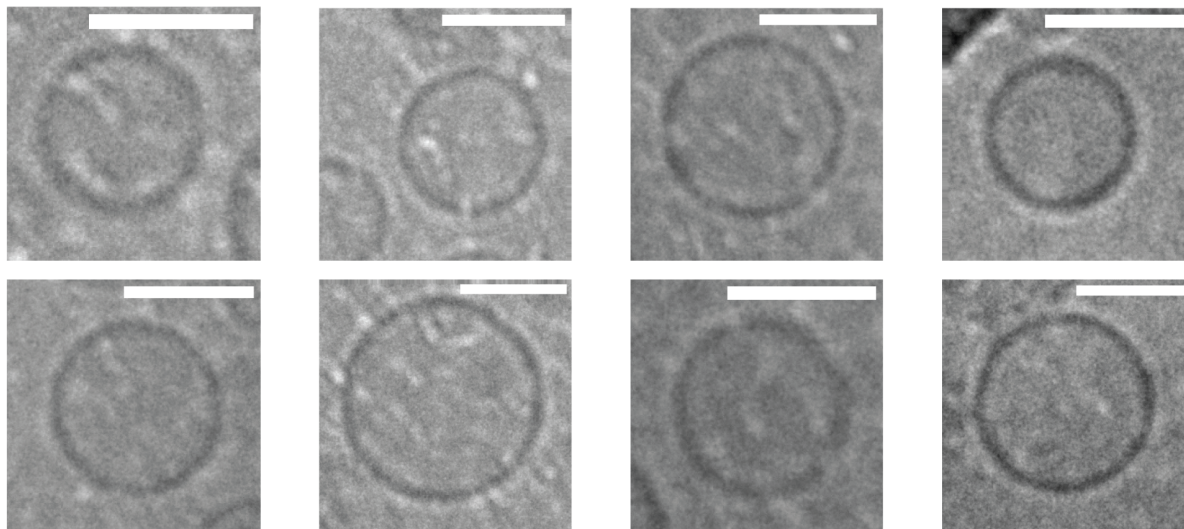
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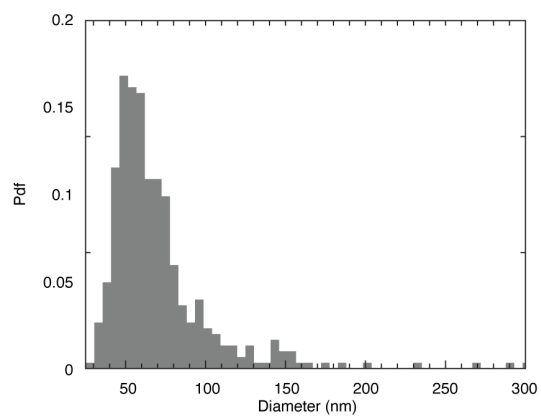
Supplementary Fig. 1 | Purification and morphology of SVs.

a, Protocol diagram for the purification of SVs. **b**, Cryo-electron micrographs of purified synaptic vesicles. SV fraction was highly pure with larger organelles rarely found in the micrographs. Data shown here was collected from multiple micrographs of a single experimental run. Scale bar is 40 nm. **c**, Distribution of SV diameters from cryo-electron micrographs. Population of SVs was found to be monodisperse with a highly conserved mean vesicle diameter of 41 ± 8 nm.

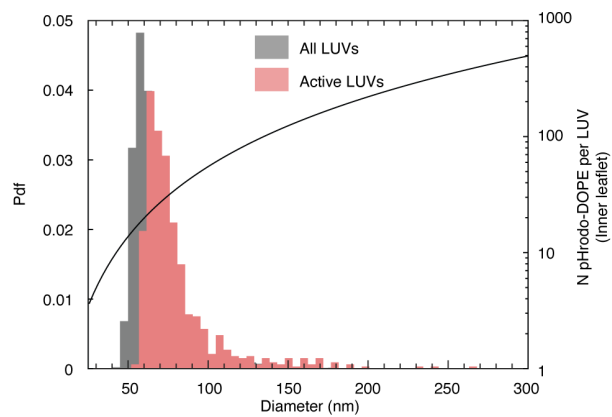
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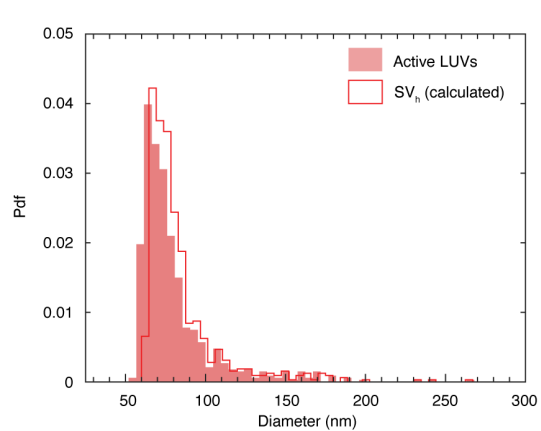
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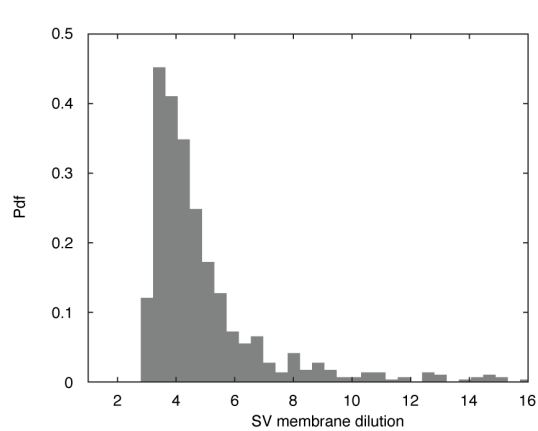
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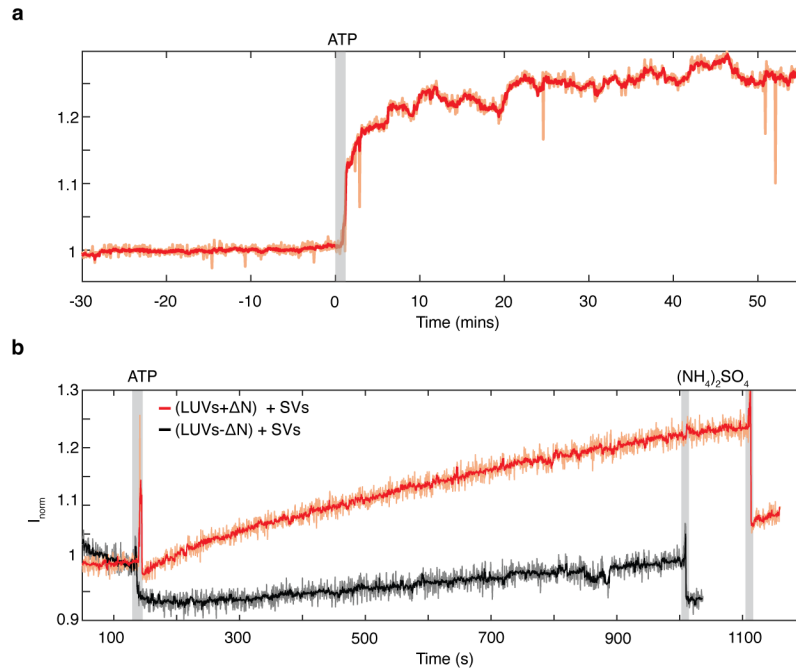


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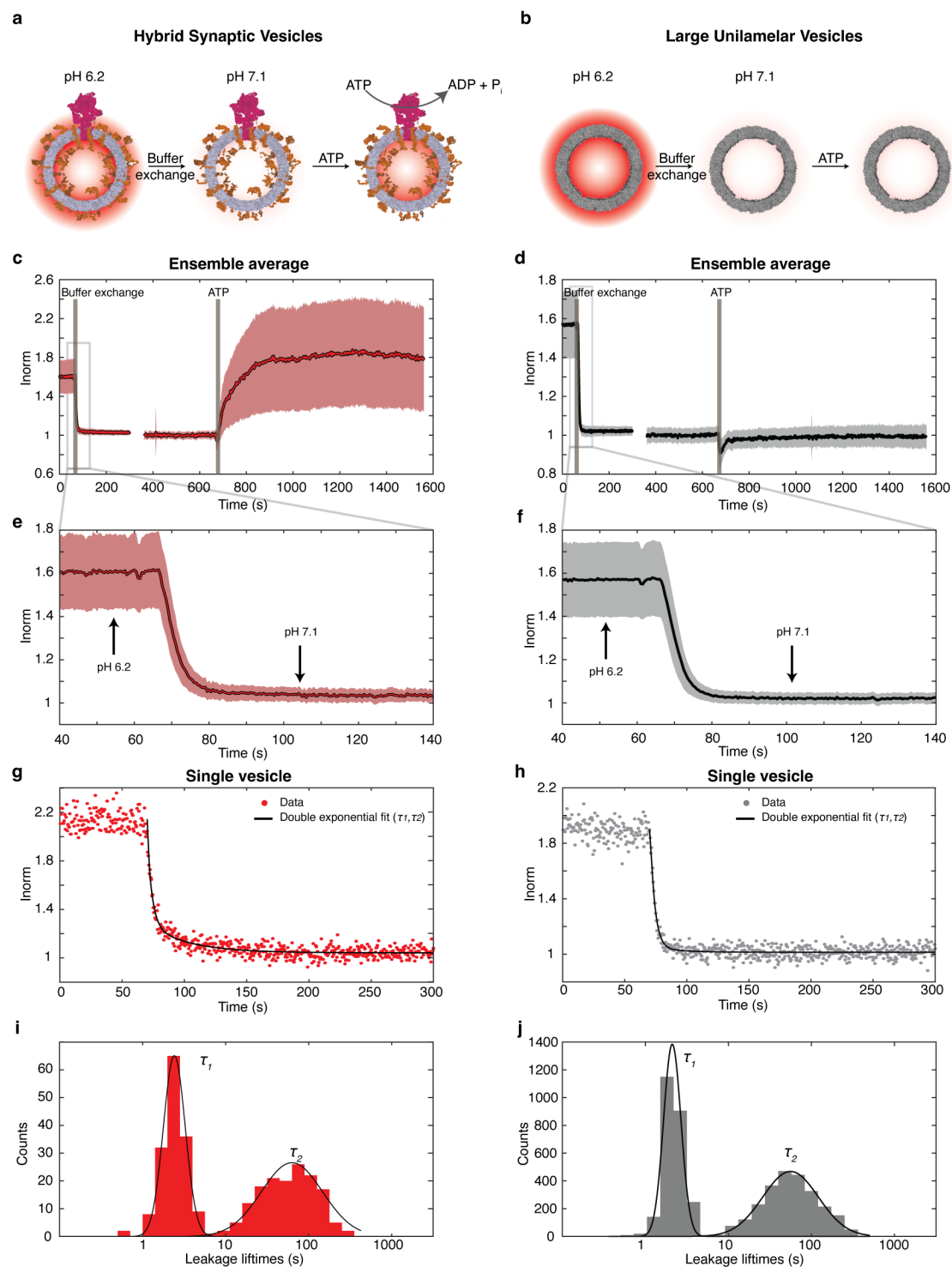
Supplementary Fig. 2 | Morphology and diameters of LUVs; number of fluorophores in LUVs; SV fold-dilution upon fusion with a LUV.

a, Cryo-electron micrographs of LUVs reconstituted with the SNARE acceptor complex ΔN . Data shown here was collected from multiple micrographs of a single experimental run. Scale bars are 50 nm. **b**, Distribution of diameters of LUVs as measured by cryoEM. The mean and SD of the distribution is 72 ± 43 nm. **c**, The cryo-electron data can be used to calibrate the vesicle size in the fluorescence microscopy images using previously established methods^{1,2} (Supplementary discussion). Grey shaded histogram shows single LUV fluorescent intensities quantitatively converted to diameter in nanometres, 66 ± 18 nm. Red shaded histogram corresponds to the size distribution of the $\sim 5\%$ of LUVs that showed V-ATPase activity, 80 ± 25 nm. The average diameter of active LUVs is ~ 14 nm larger than the rest of the population, since larger LUVs fuse more efficiently via SNARE machinery⁸. Black line corresponds to the estimated number of pHrodo-DOPE lipids in LUVs and scales with the surface area of the vesicle. The number of pHrodo-DOPE lipids in the membrane of fused vesicles ranged between 16-340 and was ~ 40 on the average. To calculate the number of lipids per vesicle we used a surface area per lipid of ~ 0.65 nm² (^{9,10}). **d**, Red-shaded histogram is the same as in c, 80 ± 25 nm. Red-outlined histogram corresponds to the calculated diameters of SV_h produced by fusion of LUVs with 40 nm SVs, 84 ± 24 nm. **e**, Dilution factor of the SV membrane in SV_h calculated using a SV diameter of 40 nm. The mean SV membrane dilution is 5.4-fold.



Supplementary Fig. 3 | Long-term activity measurements in SV_h that are surface-immobilized or suspended in solution.

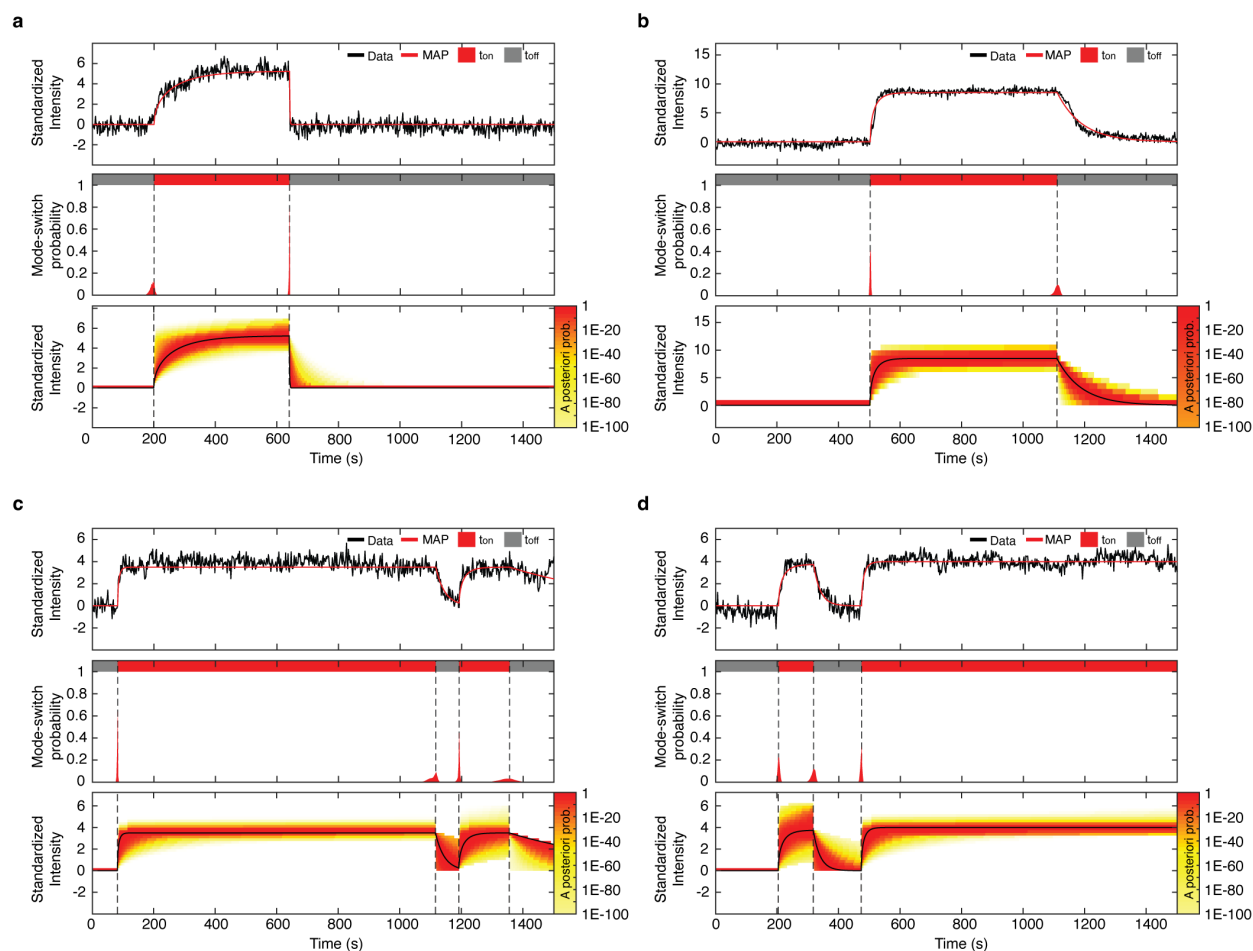
a, Activity measurements for SV_h immobilized on the glass substrate. Proton-pumping was initiated upon addition of ATP. Activity was retained for one demonstrating the biochemical stability of the V-ATPase. **b**, Activity measurements for SV_h suspended in solution. Proton pumping was initiated by addition of 2 mM ATP and stopped upon addition of 20 mM ammonium sulfate. Proteoliposomes reconstituted with the ΔN complex were able to fuse with SVs and produce SV_h that contain the V-ATPase (red trace). No fusion and therefore no activity was observed for liposomes lacking the ΔN acceptor complex (black trace). Raw data (orange and grey trace) was processed with a Chung-Kennedy filter (red and black trace)¹¹. All subsequent acidification traces shown in figures have been processed with a Chung-Kennedy filter, unless stated otherwise.



Supplementary Fig. 4 | Experimental determination of passive proton leakage in SV_h and LUVs.

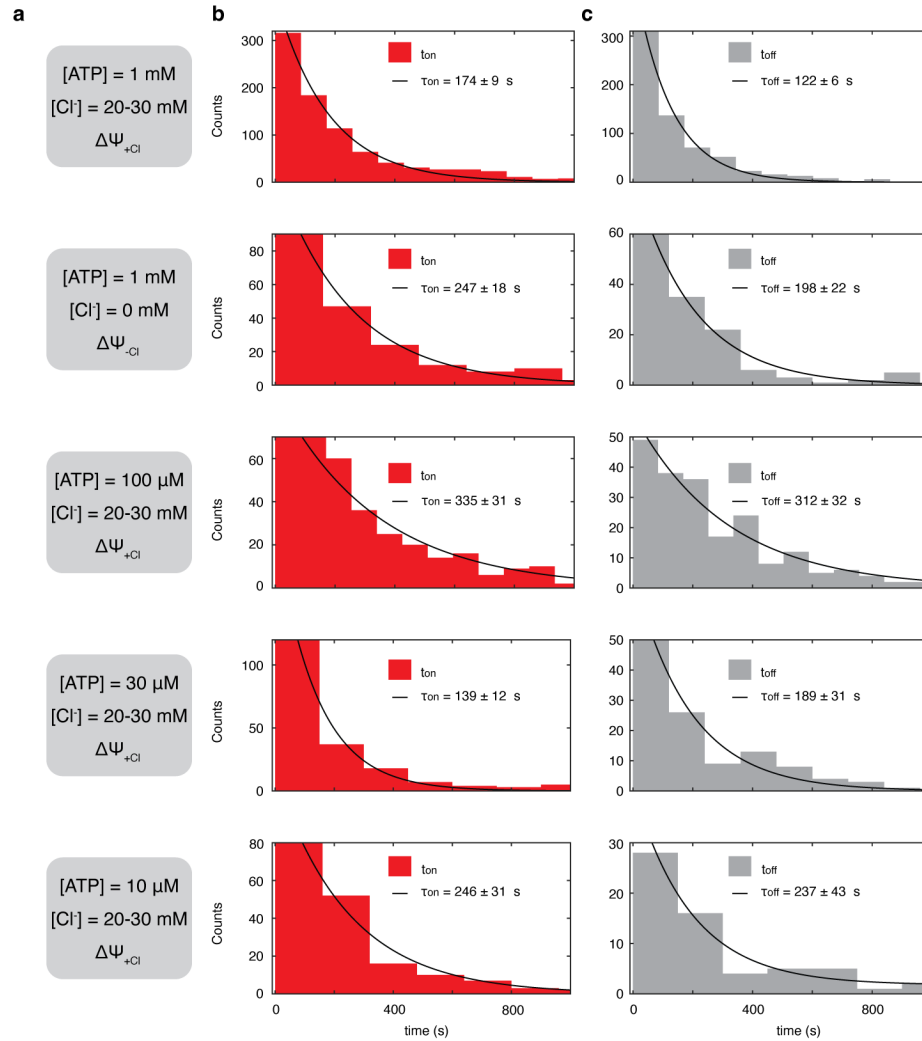
Left panels correspond to data from SV_h while right panels correspond to data from LUVs. **a, b**, Schematic illustrations of the experimental procedure. SV_h and LUVs are incubated in a glycine-

MOPS buffer at pH 6.2. Subsequently, we exchange the buffer with a glycine-MOPS buffer at *pH* 7.1 and record the change in signal intensity. Lastly, we inject ATP and record activity to differentiate between SV_h containing the V-ATPase and LUVs that don't respond to ATP. During buffer exchange, pHrodo lipids in both leaflets are subjected to the different *pH*, while during injection of ATP, only the inner leaflet of SV_h will report changes in *pH*. **c-f**, Ensemble average traces for *pH* jumps and injection of ATP. Lines correspond to the mean of the ensemble while shaded areas correspond to one standard deviation of the spread of the single vesicle data. **g, h**, Typical single vesicle kinetics of signal decrease. Data is fitted with a double exponential function to extract lifetimes of fluorescence decay for the outer leaflet (fast) and the inner leaflet (slow). **i, j**, Histograms of lifetimes derived from the double exponential fits on the single vesicle data. Gaussian fits on these histograms produced average fast and slow values. The fast component corresponding to the outer leaflet was found to be $\tau_1 = 2.41 \text{ s} \pm 1.02 \text{ s}$ and $\tau_1 = 2.11 \text{ s} \pm 1.02 \text{ s}$ for SV_h and LUVs respectively. The slow component corresponding to the inner leaflet and tracks proton leakage lifetimes was found to be $\tau_2 = 62.95 \text{ s} \pm 1.07 \text{ s}$ and $\tau_2 = 56.10 \text{ s} \pm 1.03 \text{ s}$ for SV_h and LUVs respectively. Leakage lifetimes were smoothly monotonic over time indicating a single mechanism for proton efflux.



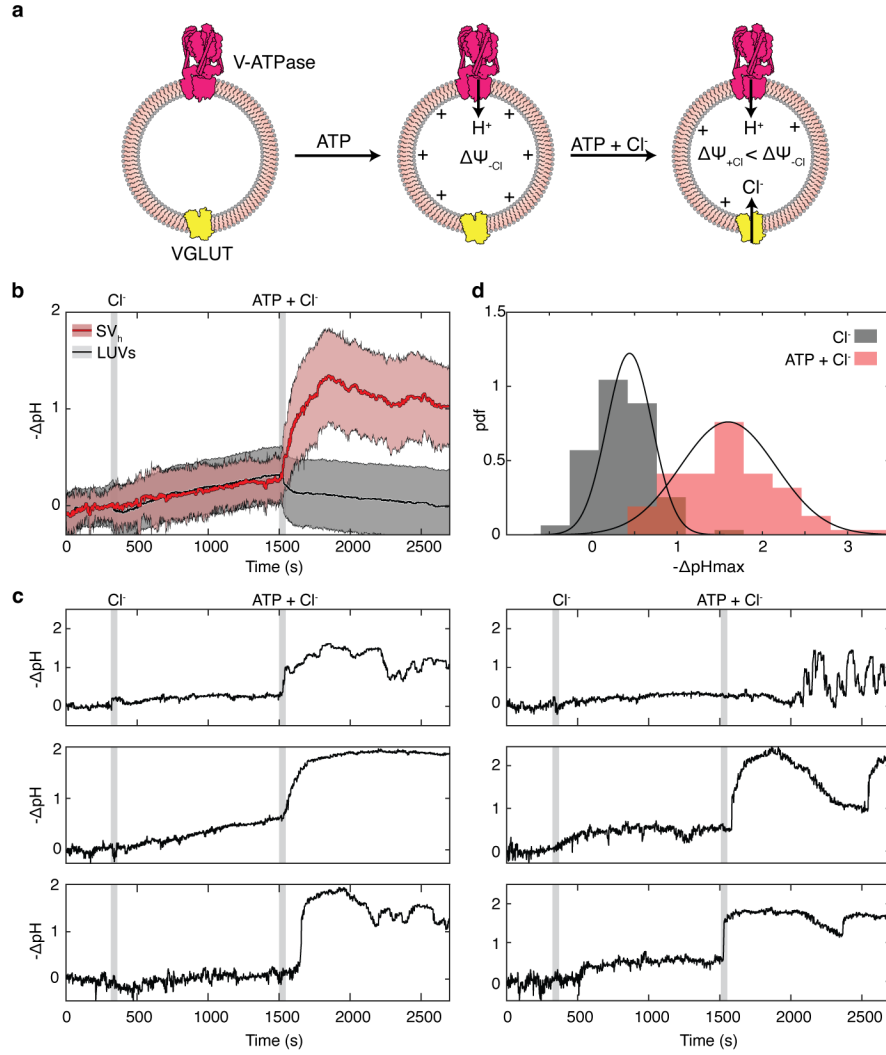
Supplementary Fig. 5 | Detection of mode-switching events and MAP fits with an exponential model.

a-d, Examples of single molecule data being analysed using stochastic filtering techniques and exponential model. The algorithm is run sequentially along observed data and then segments the data into areas where the proton pump is found in either proton-pumping or inactive states. These areas are determined using the posterior distributions for mode-switching events seen as the red distributions in the ‘mode-switching probability’ plots. When there is enough confidence (99% certainty) that an event has been observed, the data is segmented at the event time with the highest probability (in terms of the joint distribution, see Stochastic filtering section in supplementary text), shown with dashed lines. Stochastic filtering techniques are then used on the segmented data to find the parameters with the highest likelihood given the exponential model. The heatmap represents the (a posteriori) probability of where the signal is deemed to be, given the observed data and the exponential model. The MAP estimate of the signal is seen in these heatmap plots in black.



Supplementary Fig. 6 | Dwell times of the proton-pumping and inactive modes for the V-ATPase in the presence/absence of chloride and different concentrations of ATP.

Each line corresponds to data collected from different sets of experiments. **a**, Experimental conditions during which dwell times of states of the V-ATPase were extracted. **b**, Distributions of proton-pumping dwell times. **c**, Distributions of inactive dwell times. Note: any events that were interrupted by the end of the recording, were discarded. Only inactive modes after the initiation of the first pumping event were considered in the data. $n = 4-8$.

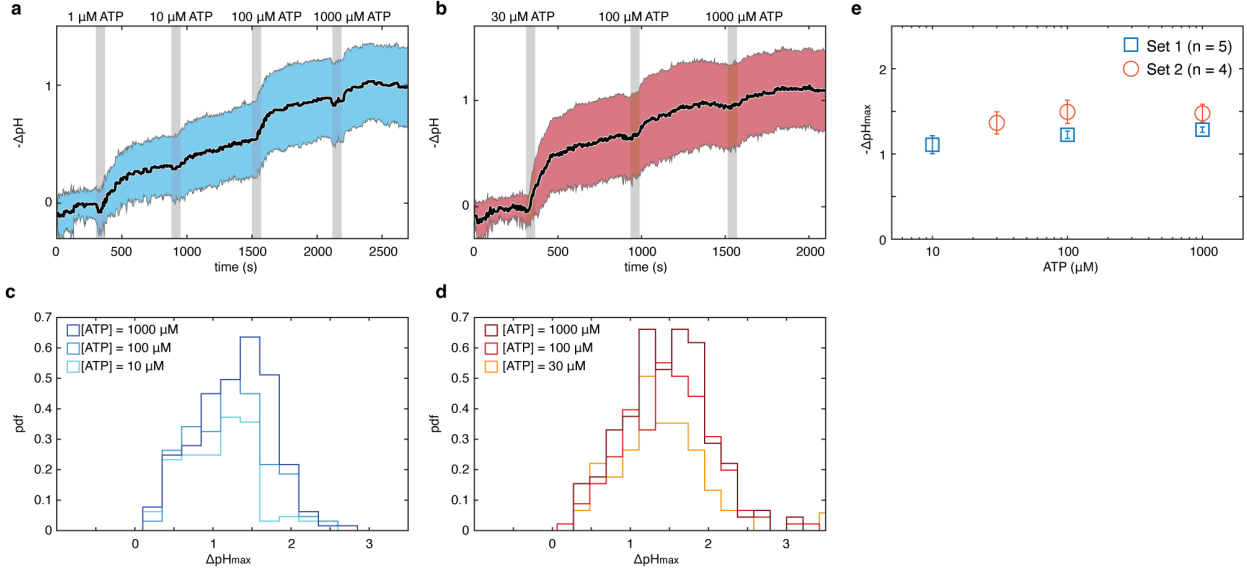


Supplementary Fig. 7 | Luminal chloride reduces membrane potential and induces mild acidification in SV_h without introducing stochastic effects.

a, Schematic illustration of membrane potential buildup during activity measurements. Upon addition of ATP, protons are pumped into the vesicles and an inside positive $\Delta\psi_{-Cl}$ is established. When extraluminal chloride is introduced, Cl^- is transported into the lumen by VGLUT and partially releases the potential resulting in $\Delta\psi_{+Cl} < \Delta\psi_{-Cl}$. **b**, Effects of chloride on ensemble average acidification kinetics of SV_h and LUVs. After recording the baseline, 30 mM choline chloride are introduced into the system. Subsequently, 30 mM choline chloride and 1 mM Mg^{2+} and ATP are added, and for SV_h , V-ATPase specific activity is monitored. Minor acidification was observed upon addition of chloride for both LUVs and SV_h , which is attributed to passive permeation of chloride ions through the membrane. No acidification was observed for LUVs upon addition of ATP. Shaded areas correspond to one SD. **c**, Typical single molecule traces for SV_h showcasing the behavior of the pump under these conditions. Addition of chloride only does not introduce any discernible stochastic effects in the form of acidification spikes. Contrary, addition of ATP introduces stochastic fluctuations in acidification levels. **d**, Histogram of the maximal acidification

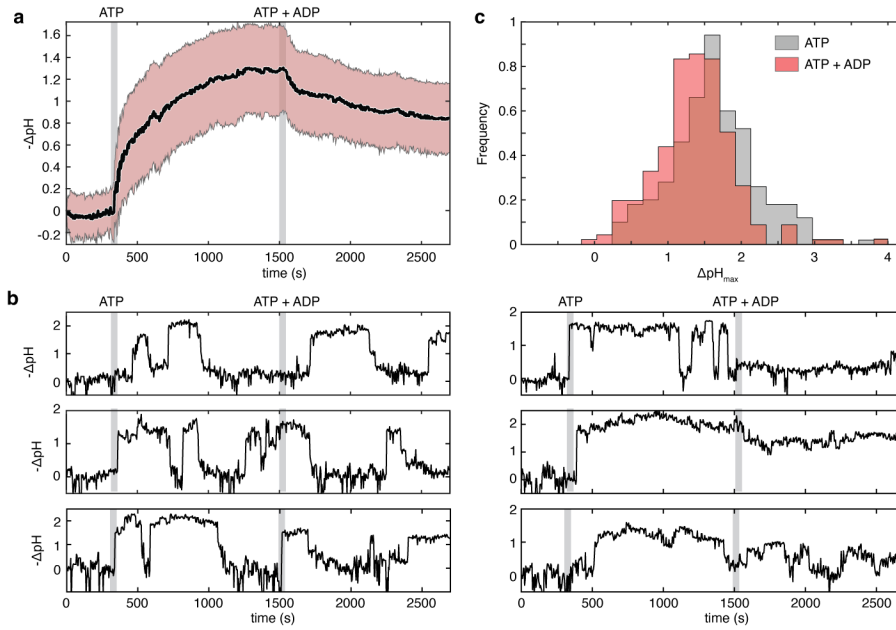
438 of SV_h , $-\Delta pH_{max}$, under the aforementioned conditions. When only chloride is present, the average
439 $-\Delta pH_{max} = 0.35$, while in the presence of both chloride and ATP, $-\Delta pH_{max} = 1.76$. Black dots
440 correspond to independent experiments performed.

441



Supplementary Fig. 8 | Additional data for ATP titration experiments.

a, b, Ensemble average acidification kinetics for two different sets of experiments with varying ATP concentrations. Black lines correspond to the mean, while blue and red shaded areas correspond to standard deviation of the single vesicle data. Note: **(b)** is also shown in Fig. 4a. **c, d**, Population histograms displaying maximal acidification plateaus (ΔpH_{max}) of the same single vesicles under different concentrations of ATP. In **(c)**, maximum acidification of vesicles when $[ATP] = 1 \mu M$ were not included. Pairwise two-sample Kolmogorov-Smirnov test showed for **(c)** statistical significance between $[ATP] = 10 \mu M$ and either $[ATP] = 100$ (*) or $[ATP] = 1000$ (***) . No statistical significance was observed between $[ATP] = 100 \mu M$ and $[ATP] = 1000 \mu M$ (n.s.). For **(d)**, pairwise two-sample Kolmogorov-Smirnov tests showed no statistical significance for either of the pairs of ATP concentrations (n.s.). **e**, Combined mean population values of $-\Delta pH_{max}$ of the histograms shown in **(c)** and **(d)** as a function of ATP concentration. Error bars correspond to SD of the mean of the independent experiments. A one-way ANOVA test gave a P -value < 0.05 (*) for set 1 (blue) and a P -value > 0.05 (n.s.) for set 2 (red). Note: data in Fig. 4c are the averaged values of set 1 and set 2 data combined.



Supplementary Fig. 9 | Additional data for ATP/ADP competition experiments.

a, Ensemble average acidification kinetics. Activity was initiated upon addition of 100 μM of ATP. Subsequently, 200 μM ADP were added to the liposomal suspension. Experiments were done in the presence of 5 mM phosphate. Black line corresponds to the mean, while shaded area corresponds to the spread of single vesicle data. **b**, Single vesicle acidification traces. **c**, Population histograms of the maximal acidification, $-\Delta\text{pH}_{\text{max}}$, of the same single vesicles in the presence of only ATP or both ATP and ADP. A two-sample Kolmogorov-Smirnov test gave a P -value < 0.001 (***)). Total number of independent experiments was $n = 3$.