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# Single Liposome Measurements for the Study of Proton-Pumping Membrane Enzymes Using Electrochemistry and Fluorescent Microscopy

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► **To cite this version:**

Ievgen Mazurenko, Nikos S Hatzakis, Lars J.C. Jeuken. Single Liposome Measurements for the Study of Proton-Pumping Membrane Enzymes Using Electrochemistry and Fluorescent Microscopy. *Journal of visualized experiments: JoVE*, 2019, 144, 10.3791/58896 . hal-02465822

**HAL Id: hal-02465822**

**<https://hal.science/hal-02465822>**

Submitted on 4 Feb 2020

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1 **TITLE:**

2 Single Liposome Measurements for the Study of Proton-Pumping Membrane Enzymes Using  
3 Electrochemistry and Fluorescent Microscopy

4  
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19  
20 **KEYWORDS:**

21 cytochrome *bo*<sub>3</sub>; single enzyme; proteoliposomes; pH-sensitive dye; proton translocation;  
22 bioelectrochemistry

23  
24 This document is the unedited Author's version of a Submitted Work that was subsequently  
25 accepted for publication in Journal of Visualized Experiments after peer review. To access the  
26 final edited and published work see [https://www.jove.com/video/58896/single-liposome-](https://www.jove.com/video/58896/single-liposome-measurements-for-study-proton-pumping-membrane)  
27 [measurements-for-study-proton-pumping-membrane](https://www.jove.com/video/58896/single-liposome-measurements-for-study-proton-pumping-membrane)

28  
29  
30 **SUMMARY:**

31 Here, we present a protocol to study the molecular mechanism of proton translocation across  
32 lipid membranes of single liposomes, using cytochrome *bo*<sub>3</sub> as an example. Combining  
33 electrochemistry and fluorescence microscopy, pH changes in the lumen of single vesicles,  
34 containing single or multiple enzyme, can be detected and analyzed individually.

35  
36 **ABSTRACT:**

37 Proton-pumping enzymes of electron transfer chains couple redox reactions to proton  
38 translocation across the membrane, creating a proton-motive force used for ATP production. The  
39 amphiphilic nature of membrane proteins requires particular attention to their handling, and  
40 reconstitution into the natural lipid environment is indispensable when studying membrane  
41 transport processes like proton translocation. Here, we detail a method that has been used for  
42 the investigation of the proton-pumping mechanism of membrane redox enzymes, taking  
43 cytochrome *bo*<sub>3</sub> from *Escherichia coli* as an example. A combination of electrochemistry and  
44 fluorescence microscopy is used to control the redox state of the quinone pool and monitor pH

45 changes in the lumen. Due to the spatial resolution of fluorescent microscopy, hundreds of  
46 liposomes can be measured simultaneously while the enzyme content can be scaled down to a  
47 single enzyme or transporter per liposome. The respective single enzyme analysis can reveal  
48 patterns in the enzyme functional dynamics that might be otherwise hidden by the behavior of  
49 the whole population. We include a description of a script for automated image analysis.

50

## 51 **INTRODUCTION:**

52 Information about enzyme mechanisms and kinetics is usually obtained on the ensemble or  
53 macroscale level with enzyme population in the thousands to millions of molecules, where  
54 measurements represent a statistical average. It is known, however, that complex  
55 macromolecules such as enzymes may demonstrate heterogeneity in their behavior and  
56 molecular mechanisms observed at the ensemble level are not necessarily valid for every  
57 molecule. Such deviations on the individual molecule scale have been extensively confirmed by  
58 studies of single enzymes with a variety of methods emerging during the last two decades<sup>1</sup>.  
59 Notably, fluorescence detection of individual enzyme activity has been used to investigate  
60 heterogeneity of enzymes activity<sup>2,3</sup> or discover the so-called memory effect (periods of high  
61 enzymes activity succeeded by periods of low activity and *vice versa*)<sup>4,5</sup>.

62

63 Many single enzyme studies require that the enzymes are immobilized on the surface or spatially  
64 fixed in another way to remain sufficiently long in the field of view for continuous observation.  
65 Enzyme encapsulation into liposomes has been shown to enable enzyme immobilization while  
66 preventing any negative impact due the surface-enzyme or protein-protein interactions<sup>6,7</sup>. In  
67 addition, liposomes offer a unique possibility to study single membrane proteins in their natural  
68 lipid bilayer environment<sup>8-10</sup>.

69

70 A class of membrane proteins, transporters, exercises a directional translocation of substances  
71 across the cell membrane, a behavior that can only be studied when proteins are reconstituted  
72 into the lipid bilayers (*e.g.*, liposomes)<sup>11-13</sup>. For example, proton translocation, exhibited by  
73 several enzymes of prokaryotic and eukaryotic electron transport chains, plays an important role  
74 in cellular respiration by creating a proton-motive force used for ATP synthesis. In this case, the  
75 proton pumping activity is coupled to the electron transfer, although the detailed mechanism of  
76 this process often remains elusive.

77

78 Recently, we demonstrated the possibility to couple fluorescent detection with electrochemistry  
79 to study proton pumping activity of single enzymes of the terminal ubiquinol oxidase of  
80 *Escherichia coli* (cytochrome *bo*<sub>3</sub>) reconstituted in the liposomes<sup>14</sup>. This was achieved by  
81 encapsulation of a pH-sensitive membrane-impermeable fluorescent dye into the lumen of  
82 liposomes prepared from *E. coli* polar lipids (**Figure 1A**). The protein amount was optimized so  
83 that most liposomes either contained no or only one reconstituted enzyme molecule (according  
84 to Poisson distribution). The two substrates of cytochrome *bo*<sub>3</sub> were provided by adding  
85 ubiquinone to the lipid mix that formed the liposomes and (ambient) oxygen in solution. The  
86 liposomes are then sparsely adsorbed on a semi-transparent ultra-smooth gold electrode,  
87 covered with a self-assembled monolayer of 6-mercaptohexanol. Finally, the electrode is  
88 mounted on the bottom of a simple spectroelectrochemical cell (**Figure 1B**). Electrochemical

89 control of the quinone pool redox state allows one to flexibly trigger or to stop the enzymatic  
90 reaction at any moment, while the pH-sensitive dye is used to monitor pH changes inside the  
91 lumen of the liposomes as a result of proton translocation by the enzymes. By using the  
92 fluorescence intensity of a second, lipid-bound fluorescent dye, the size and volume of individual  
93 liposomes can be determined and thus the quantification of enzyme proton pumping activity.  
94 Using this technique, we notably found that cytochrome *bo*<sub>3</sub> molecules are able to enter into a  
95 spontaneous leak state that rapidly dissipates the proton motive force. The goal of this article is  
96 to introduce the technique of single liposome measurements in detail.

97

## 98 **PROTOCOL:**

99

### 100 **1. Preparation of an *E. coli* Polar Lipids Mix**

101

102 NOTE: *E. coli* lipids used for the liposomes' preparation should be aliquoted and thoroughly mixed  
103 with ubiquinone-10 (enzyme substrate) and long-wavelength fluorescent dye-labeled lipids (for  
104 liposomes size determination) prior to the reconstitution.

105

106 1.1. Using a glass syringe, transfer 200  $\mu$ L of chloroform stock of lipid polar extract from  
107 *Escherichia coli* (25 mg/mL) into glass vials to make 5 mg aliquots.

108

109 1.2. Add 50  $\mu$ L of 1 mg/mL ubiquinone-10 (UQ-10; in chloroform) to the lipids to make the  
110 final ratio of UQ-10: lipids 1:100 (1% w/w).

111

112 1.3. Add 20  $\mu$ L of 1 mg/mL (0.4% w/w) of a long-wavelength fluorescent dye-labeled lipid  
113 (FDLL) to the lipids/UQ-10 mix.

114

115 1.4. Homogenize the chloroform solution by short vortexing and evaporate most of the  
116 chloroform under a gentle nitrogen or argon flow. Remove the chloroform traces entirely by  
117 further evaporation under vacuum for at least 1 h.

118

119 NOTE: Lipid aliquots can be stored under an inert atmosphere at -20  $^{\circ}$ C for several months.

120

### 121 **2. Reconstitution of Cytochrome *bo*<sub>3</sub>**

122

123 NOTE: For the purification of cytochrome *bo*<sub>3</sub> from *E. coli*, follow the protocol from Rumbley *et*  
124 *al.*<sup>15</sup> To ensure high purity of natively-folded enzyme samples, add size-exclusion  
125 chromatography after the affinity purification step described by Rumbley *et al.*<sup>15</sup>

126

127 2.1. Add 312.5  $\mu$ L of 40 mM MOPS-KOH/60 mM K<sub>2</sub>SO<sub>4</sub>, pH 7.4, to one aliquot of lipids/UQ-  
128 10/FDLL dry mix (5 mg, step 1.4) and re-suspend using vortex mixing followed by 2 min of  
129 treatment in an ultrasonic bath.

130

131 2.2. Add 125  $\mu$ L of 25 mM 8-hydroxypyrene-1,3,6-trisulphonic acid (HPTS), a pH-sensitive  
132 fluorescent dye that needs to be encapsulated inside the liposomes.

133

134 2.3. Add 137.5  $\mu\text{L}$  of 250 mM n-octyl  $\beta$ -D-glucopyranoside (OGP) surfactant, mix using vortex  
135 and sonicate in an ultrasonic water bath for 10 min to ensure all lipids are solubilized into  
136 surfactant micelles. Transfer the dispersion into a 1.5 mL plastic tube.

137

138 NOTE: Cloudy suspension of lipids should become transparent after the solubilization with  
139 surfactant.

140

141 2.4. Add the required amount of cytochrome  $b_{o_3}$  (see the note below) and add ultrapure  
142 water to make a total volume of 50  $\mu\text{L}$  (cytochrome  $b_{o_3}$  solution plus water). Incubate at 4  $^{\circ}\text{C}$  for  
143 10 min on a roller mixer.

144

145 NOTE: Typical amount for single enzyme conditions is 0.1 – 0.2% (w/w protein-to-lipid, *i.e.* 5 – 10  
146  $\mu\text{g}$  of protein), although it can be increased till 1 – 2% (50 – 100  $\mu\text{g}$  of protein) if the goal is to  
147 observe only the electrochemical activity (see below). As a negative control, liposomes without  
148 cytochrome  $b_{o_3}$  can be prepared.

149

150 2.5. Weigh 2x 50 mg and 2x 100 mg of polystyrene microbeads into four 1.5 mL-tube caps and  
151 close with paraffin film to prevent drying.

152

153 NOTE: Before use, polystyrene microbeads should be washed with methanol, water and stored  
154 in water according to the manufacturer's manual.

155

156 2.6. Add the 1<sup>st</sup> **50 mg** of polystyrene microbeads into the reconstitution mixture (step 2.4) by  
157 putting the cap with polystyrene microbeads on the 1.5 mL-plastic tube with dispersion and  
158 performing a short spin for a couple of seconds. Incubate at 4  $^{\circ}\text{C}$  on a roller mixer for polystyrene  
159 microbeads to adsorb the surfactant for **30 min**.

160

161 2.6.1. Repeat the additions of polystyrene microbeads and incubations as follows: add **50 mg** of  
162 microbeads for **60 min** of incubation; add **100 mg** of microbeads for **60 min** of incubation; and  
163 add **100 mg** of microbeads for **120 min** of incubation.

164

165 NOTE: The solution above the settled microbeads will turn translucent during step 2.6 as  
166 proteoliposomes are formed.

167

168 2.7. Separate the proteoliposome solution from the polystyrene microbeads using a  
169 micropipette with a thin tip. Dilute the dispersion in 90 mL of 20 mM MOPS-KOH/30 mM  $\text{K}_2\text{SO}_4$ ,  
170 pH 7.4 (MOPS buffer) and transfer in a Ti45 ultracentrifuge tube.

171

172 2.8. Ultracentrifuge the dispersion using Type 45 Ti rotor at 125,000 x g (at  $r_{\text{max}}$ ) for 1 h to  
173 pellet the proteoliposomes.

174

175 NOTE: Smaller centrifuge tubes can be used although the dilution in large buffer volume helps to  
176 reduce the concentration of the non-encapsulated HPTS in the final suspension.

177  
178 2.9. Discard the supernatant, rinse the pellets with 20 mM MOPS-KOH/30 mM K<sub>2</sub>SO<sub>4</sub>, pH 7.4  
179 buffer (without resuspending the pellet). Then re-suspend the proteoliposomes in 500 μL of  
180 MOPS buffer by pipetting it back and forth with thin tip micropipette. Then transfer to a 1.5 mL  
181 plastic tube.

182  
183 2.10. Centrifuge the suspension for 5 min at 12,000 x g to remove the debris. Transfer the  
184 supernatant (reconstituted proteoliposomes) into a new vial.

185  
186 2.11. Store the reconstituted proteoliposomes dispersion at 4 °C overnight and use within 2  
187 days.

### 188 189 **3. Fabrication of Semi-Transparent Gold Electrodes**

190  
191 NOTE: The smooth gold surface is obtained by a template stripping method of a 30 nm-thick layer  
192 of 99.99% gold from an atomically smooth silicon wafer. The small thickness of the gold layer is  
193 important since it must be semi-transparent to permit fluorescence observation. Details of gold  
194 evaporation (physical vapor deposition, PVD) can be find elsewhere<sup>16</sup> and only template-  
195 stripping is covered here. Alternatively, ultra-smooth gold chips can be purchased elsewhere (see  
196 **Table of Materials**).

197  
198 3.1. Glue up to 9 glass cover slips (0.17 mm thick) onto the evaporated gold surface using bi-  
199 component low-fluorescence epoxy. Cure the glue at 80 °C for 4 h.

200  
201 3.2. Just before modification with a self-assembled monolayer (step 4), detach the glass cover  
202 slips from the silicon wafers with a blade. Due to the thinness of the cover slips, take care when  
203 detaching the cover slips to not crack or break the glass slides.

### 204 205 **4. Modification of the Gold Surface with Self-Assembled Monolayer (SAM)**

206  
207 4.1. Prepare 5 mL of water solution of 1 mM 6-mercaptohexanol (6MH).

208  
209 4.2. Dip freshly detached gold-coated cover slips (step 3.1) into 6MH solution and leave at 20  
210 – 25 °C overnight (>16 h) to form the SAM.

211  
212 NOTE: Thiol solutions have an unpleasant smell, so a closed vessel should be used when  
213 incubating the gold-coated cover slip.

214  
215 4.3. The next day, remove the gold-coated cover slip from the 6MH solution, wash briefly with  
216 water or methanol and then with isopropanol. Dry under a gentle gas flow.

### 217 218 **5. Electrochemical Testing of Proteoliposomes Activity**

219  
220 NOTE: The electrochemical activity of enzyme is first verified on a closely packed liposomes layer

221 (step 5) and lower vesicle coverages are used in single vesicles experiment to measure pH  
222 changes in the lumen of the liposomes (step 6).

223  
224 5.1. Assemble the gold-coated cover slip in a spectro-electrochemical cell (see **Figure 1**). Make  
225 contact to the gold with a flat wire outside an area defined by a rubber O-ring.

226  
227 5.2. Add 2 mL of the electrolyte buffer solution and place the reference and auxiliary  
228 electrodes in the cell.

229  
230 NOTE: As further discussed in the Discussion section, references electrodes without chloride are  
231 preferred to prevent formation of Au(I)Cl during electrochemistry. Here, a Hg/Hg<sub>2</sub>SO<sub>4</sub> (sat. K<sub>2</sub>SO<sub>4</sub>)  
232 reference electrode is used and potentials are given versus Standard Hydrogen Electrode (SHE)  
233 using 0.658 V vs SHE for the Hg/Hg<sub>2</sub>SO<sub>4</sub> (sat. K<sub>2</sub>SO<sub>4</sub>).

234  
235 5.3. Run electrochemical impedance spectroscopy (0.1 Hz – 100 kHz) at open cell potential  
236 (OCP) potential to assess the quality of SAM. Convert impedance values to admittance and divide  
237 by  $2\pi\omega$  to plot a Cole-Cole plot, where  $\omega$  is the frequency (see the following references<sup>16–18</sup> for  
238 details on converting and interpreting impedance spectra).

239  
240 NOTE: Compact and dense SAMs of 6MH should give a close to semi-circle Cole-Cole plot and  
241 give capacitance values in the range 2.5-3.0  $\mu\text{F}/\text{cm}^2$  (**Figure 2A**). If significant deviation from semi-  
242 circle shape or out-of-range capacitance values are obtained, change the electrode.

243  
244 5.4. Run blank cyclic voltammograms (CVs) with scan rates 100 and 10 mV/s in the potential  
245 region -0.3 – 0.8 V. A typical CV is shown on (**Figure 2B**, dashed line).

246  
247 NOTE: This should demonstrate almost pure capacitive behavior and an absence of significant  
248 faradaic current, even under ambient oxygen conditions as used here.

249  
250 5.5. Add proteoliposomes (0.5 mg/mL final lipids concentration, 1-2% (w/w) ratio of  
251 cytochrome *bo*<sub>3</sub> to lipid) to the electrochemical cell and mix slightly with a pipette. Wait until the  
252 adsorption of proteoliposomes on the electrode surface is finished (30-60 min at room  
253 temperature).

254  
255 NOTE: Cyclic voltammograms (CVs) at 10 mV/s can be run during the proteoliposomes adsorption  
256 to follow the process. The adsorption is finished when consecutive CVs stop changing.  
257 Information about CV techniques can be found in the following textbooks.<sup>19, 20</sup>

258  
259 5.6. Wash the cell by changing the buffer solution at least 10 times but avoid leaving the  
260 electrode surface completely dry.

261  
262 5.7. Run the electrochemical impedance spectroscopy at OCP (**Figure 2A**, blue line) to confirm  
263 the SAM on the gold electrode remains unchanged and CVs with scan rates 10 and 100 mV/s to  
264 observe catalytic ubiquinol oxidation (and oxygen reduction) by cytochrome *bo*<sub>3</sub> at onset

265 potentials of electrochemical quinone reduction about 0 V vs SHE) (**Figure 2B**).

266

## 267 **6. Detection of Enzymatic Proton Pumping by Fluorescence Microscopy**

268

269 6.1. Modify the gold electrode as in step 5 but using 100x less proteoliposomes compared to  
270 step 5.5 (*i.e.*, 5 µg/mL). For single enzyme studies, reduce the cytochrome *bo*<sub>3</sub> to lipid ratio to  
271 0.1-0.2% (w/w).

272

273 NOTE: Liposomes will sparsely adsorb on the electrode surface enabling single vesicle monitoring  
274 by fluorescence microscopy. Under these single-enzyme conditions, the amount of enzyme  
275 immobilized on the electrode surface is insufficient for the observation of a catalytic current.

276

277 6.2. Place the electrochemical cell on the oil objective (60X) of an inverted fluorescence  
278 microscope with a drop of immersion oil. Using appropriate filters for FDLL fluorescence, focus  
279 on the electrode surface. Single liposomes should appear as bright spots at the diffraction limit  
280 of the microscope/objective. Take an image of FDLL fluorescence.

281

282 6.3. Switch to the one of HPTS fluorescence filter sets on the microscope to verify that HPTS  
283 fluorescence is clearly visible and distinguishable from the background (at the chosen exposure  
284 time, see step 6.4). Increase the light intensity of the lamp source if it is not the case.

285

286 6.4. Program the microscope software to perform a timed image acquisition by alternating  
287 two HPTS filter sets (menu **Applications | Define/Run ND Acquisition**). Set the delay between  
288 image acquisitions at minimum. In this experiment, use a 1 s exposure and 0.3 s delay (due to the  
289 turret movement).

290

291 NOTE: The ratio between fluorescence intensities at these two channels will be later converted  
292 to pH inside the liposomes at each time point. The duration of the acquisition can vary according  
293 to the expected pH change rate; 5 min is used in this article.

294

295 6.5. Adjust the settings of the potentiostat to change the potential during the image  
296 acquisition. For example, in this experiment, use the following sequence: 0 – 60 s: no potential  
297 applied (*i.e.* OCP); 60 – 180 s: -0.2 V (vs SHE); 180 – 300 s: 0.4 V (vs SHE). In this case, only the  
298 applied potential in the second phase (-0.2 V vs SHE) is sufficient to efficiently reduce the quinone  
299 pool.

300

301 6.6. Run simultaneously the timed images acquisition (microscope) and the potential  
302 sequence (potentiostat) by manually starting both measurements at the same time.

303

304 NOTE: The experiment can be repeated on the same electrode several time by moving the  
305 microscope stage to a different area on the surface. The delay between the acquisitions should  
306 be at least 5-10 min to insure complete pH equilibration of liposomes on the surface. Different  
307 durations and potential patterns can be applied depending on the need, although imaging time  
308 is limited due to photobleaching of HPTS during acquisition.



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## 7. Analysis of Fluorescence Images

NOTE: A typical experiment produces a set of images with a time step (*e.g.*, 2.6 s) for each of the two channels, *i.e.*, (duration \* 2 / 2.6) images. An example of such image set recorded during a single enzyme experiment can be accessed via the Research Data Leeds repository<sup>21</sup>. An image treatment consists of several steps by using Fiji (ImageJ) and high-level mathematical analysis programming language software (henceforth referred to as scripting software, see **Table of Materials** for details).

7.1. Use Fiji to separate time lapse file, align images and save as separate channels and timeframes.

7.1.1. Open time lapse file using the Bioformats importer provided within Fiji (macro command: run ("Bio-Formats Importer")).

7.1.2. Use the plugin StackReg to align each frame to the first one to account for possible stage movements or thermal drift occurred during the acquisition (macro command: run("StackReg ", "transformation=Translation")).

7.1.3. Save separate uncompressed image files for each channel and each time step in TIFF format into a single folder.

7.1.4. Extract the exact time stamps for each frame from the time lapse file metadata and save them as a CSV-file in the same folder as the images. Alternatively, extract time stamps using the acquisition software and save manually into a CSV-file using a spreadsheet software.

NOTE: The folder with images and time stamps is now ready to be analyzed by the scripting software. Steps 7.1.1. – 7.1.4. can be automatized using a Fiji script (a script written in Python for batch processing of time lapse files is provided, use “**Ctrl-Shift-N**” (in Windows), then **File | Open** to load the script).

7.2. Use scripting software for automatized processing of the images. Load the provided code to the software and click **Run to end**. When prompted, select the folder containing the images from step 7.1.

NOTE: A script for analysis is provided as mlx-format live script, with extensive comments, to identify single liposomes, fit them to 2D-Gaussian function, filter them and quantify the pH values at each point of time. The following sub-steps are executed automatically by the script.

7.2.1. Load all time frames images for a single experiment into the memory.

7.2.2. Average all images for a single channel (select the channel with the highest fluorescence intensity) and use the averaged image to identify all maximums that might correspond to the

353 single liposomes.

354

355 7.2.3. Fit the identified maximums on the averaged image to the 2D-Gaussian function and save  
356 the resulted fit parameters for each liposome (see **Figure 4A** for fitted liposome example).

357

358 7.2.4. Filter the maximums according to the expected single liposomes criteria, such as size,  
359 circularity and intensity. Reject liposomes that are poorly fitted due to low signal to noise, closely  
360 neighboring liposome or being too close to the image edge.

361

362 7.2.5. Load time stamps from the external file and fit each filtered liposome to the 2D-gaussian  
363 function on each time frame image separately.

364

365 NOTE: The use of parallel programming and multicore CPU can significantly enhance the  
366 calculation speed at this stage.

367

368 7.2.6. Filter the liposomes again according to the similar criteria as in step 7.2.4 but applied to  
369 each time frame.

370

371 7.2.7. On each time step, define the fluorescence intensity ratio of a liposome as the ratio of  
372 volumes enclosed by fitted 2D-Gaussian function at two channels. Calculate the pH values from  
373 the ratio of intensities determined from the two HPTS channels and using a calibration curve  
374 (step 8). Plot resulting pH-time curves for the liposomes and observe their pH change when the  
375 potential is applied.

376

## 377 **8. Performing a Calibration Curve of HPTS Fluorescence**

378

379 NOTE: To convert HPTS fluorescence ratio to an intravesicular pH, a calibration curve must be  
380 first established that would take into account particular conditions of experiments such as gold  
381 transmittance, filters quality, *etc.* This step has to be performed only once or twice and the  
382 calibration data can be used as long as the setup and measurement parameters remain the same  
383 in step 6.

384

385 8.1. Prepare an electrode with sparsely adsorbed liposomes (without cytochrome *bo*<sub>3</sub> as  
386 described in Steps 5 and 6.1).

387

388 8.2. Add 2  $\mu$ L of 0.1 mg/mL gramicidin solution in ethanol to 2 mL of buffer to create the  
389 concentration 100 ng/mL.

390

391 8.3. Capture two fluorescence images for the two HPTS channels.

392

393 8.4. Change the pH of the cell by addition of small aliquots of 1 M HCl or 1 M H<sub>2</sub>SO<sub>4</sub>. Measure  
394 the pH of the buffer with a standard pH meter and capture two fluorescence images for the two  
395 HPTS channels.

396

- 397 8.5. Repeat steps 8.4 for the pH range 6 to 9.  
398  
399 8.6. Using the algorithm from 7.2, fit, filter and calculate the average HPTS fluorescence ratio  
400 of individual liposomes at every pH. Take the HPTS ratio average over all liposomes.  
401  
402 8.7. Fit the resulting pH-ratio dependence to the following equation:  
403 
$$pH = pK_a + \log\left(\frac{Ratio - R_a}{R_b - Ratio}\right)$$
, where  $pK_a$ ,  $R_a$  and  $R_b$  are fitting parameters.  
404  
405 8.8. Use  $pK_a$ ,  $R_a$  and  $R_b$  to convert the HPTS ratio of individual liposomes in step 7.2.7. to pH  
406 values.  
407

### 408 REPRESENTATIVE RESULTS:

409 The quality of the gold-modified cover slip (the electrode) with a SAM of 6MH is checked before  
410 each experiment with electrochemical impedance spectroscopy. **Figure 2A** shows representative  
411 Cole-Cole plots measured using electrochemical impedance spectroscopy before and after  
412 liposomes are adsorbed. If the quality of SAM is sufficient, impedance spectroscopy should  
413 demonstrate an almost pure capacitive behavior resulting in a semi-circle Cole-Cole plot. The  
414 diameter of the semi-circle in a Cole-Cole plot equals the double layer capacitance of the  
415 electrode surface, which should be in the 2.5-3.0  $\mu\text{F}/\text{cm}^2$  range. Note that the capacitance should  
416 not change significantly upon liposomes adsorption, although a slight increase in capacitance  
417 might be observed (high liposomes coverage, **Figure 2A**, bottom) or a slight shift in impedance  
418 can be observed at low frequencies (low liposome coverages, **Figure 2A**, top).  
419

420 Electrochemistry can be used to test the catalytic activity of cytochrome  $bo_3$  in the  
421 proteoliposomes, where catalytic currents measured with cyclic voltammetry reflect the  
422 ubiquinol-oxygen oxidoreductase activity. However, significant catalytic currents can only be  
423 detected at high quantities of adsorbed proteoliposomes and a closely packed layer of  
424 proteoliposomes on the gold-modified cover slip is needed. **Figure 2B** demonstrates  
425 representative cyclic voltammograms (CVs) of the electrode before and after liposomes  
426 adsorption with either low or high liposome coverage. No faradaic current is observed on the  
427 blank CV because oxygen reduction by the bare gold electrode is blocked by the SAM. When the  
428 surface is saturated with liposomes with high cytochrome  $bo_3$  content (1.3% (w/w) in this case),  
429 a clear catalytic wave due to oxygen reduction is observed with an onset potential of 0 V, *i.e.*, the  
430 potential of ubiquinone reduction (**Figure 2B**, blue line). The ubiquinone pool acts both as the  
431 natural substrate for the enzyme and as an electron mediator, transferring electrons from the  
432 enzyme to the electrode surface. We note that under high liposome coverage, no distinction of  
433 individual vesicles is possible by microscopy and lower coverage is needed for single liposome  
434 studies. At low liposome coverage (but high protein to lipid ratio), the catalytic current is  
435 significantly reduced, barely distinguishable from background (**Figure 2B**, red line). Note that  
436 under single enzyme conditions (low protein to lipid ratio), the catalytic current is even lower and  
437 cannot be measured reliably.  
438

439 **Figure 3** shows fluorescence images of liposomes adsorbed on the electrodes at three different

440 coverages. All images were taken in identical light and exposure conditions and their brightness  
441 was adjusted equally to enable direct comparison. The dye-containing-liposomes are visible on  
442 the images as bright spots. The central part of the image was photobleached for a couple of  
443 minutes to reveal background fluorescence level (we note that FDLL is relatively photostable and  
444 is not photobleached completely in **Figure 3**). The images on two HPTS channels are  
445 superimposable, where the ratio between the two channels (410/535 and 470/535) corresponds  
446 to pH 7.4 used in this experiment. A larger number of liposomes are visible with the FDLL channel,  
447 which indicates the presence of liposomes that have no HPTS encapsulated. The difference  
448 between the HPTS and FDLL channels is more pronounced at higher coverages, possibly because  
449 at high liposome coverage, liposomes are more likely to burst or fuse on the surface.

450  
451 The image analysis requires the alignment of frames (against the first frame) using the ImageJ,  
452 plugin StackReg. The alignment is indispensable in most situations since a slight thermal drift of  
453 the sample usually occurs within the timescale of the experiment, changing the liposome  
454 coordinates. All further analysis is performed using a scripting software code. This code performs  
455 automatic liposome identification. As the size of liposomes are below the Abbe diffraction limit,  
456 their fluorescence is seen as a point spread function much larger than the actual liposome  
457 diameter (**Figure 4A**). The code fits the fluorescence intensity of each vesicle on two channels to  
458 a 2D-Gaussian function (**Figure 4A**) and calculates their volumetric intensity ratio that can be  
459 converted to pH using a calibration curve. By performing these actions on all time frames, the pH  
460 is obtained inside every vesicle within timescale of the experiment (**Movie 1**). **Figure 4B** shows  
461 the medians of all vesicle pH changes within a single image when cytochrome *bo<sub>3</sub>* content is 1.3%.  
462 An increase in pH (proton pumping) is clearly visible when a potential between -0.1 and -0.3 V is  
463 applied, but not for 0 V since the latter is not sufficient to reduce the quinone pool. When  
464 cytochrome *bo<sub>3</sub>* content is much lower (protein-to-lipid ratio of 0.1%, **Figure 4C**), the median  
465 curves become almost indistinguishable from those of empty liposomes (**Figure 4D**). The  
466 difference becomes evident when considering individual pH traces of random liposomes (**Figure**  
467 **5, 6 and 7**). While liposomes without cytochrome *bo<sub>3</sub>* display no significant pH changes with  
468 respect to noise (**Figure 7**), a selection of liposomes show an increase (dominantly) or a decrease  
469 of pH when a potential is applied that activates cytochrome *bo<sub>3</sub>* (**Figures 6**, grey zone). The obvious  
470 difference in pH-traces between liposomes is consistent with the low protein-to-lipid ratio, where  
471 cytochrome *bo<sub>3</sub>* is only present in a small subset of liposomes activity. The prevalence of a pH  
472 increase over decrease is explained by the reconstitution method that favors an “outward”  
473 orientation of enzyme molecules (*ca.* 75%). The fraction of liposomes that display a pH change  
474 increases when the cytochrome *bo<sub>3</sub>* to lipid ratio is higher (**Figure 5**). Note also that some  
475 liposomes stop proton pumping and enter into proton dissipation mode before the end of the  
476 potential application. We attribute this behavior to the cytochrome *bo<sub>3</sub>* molecules entering a  
477 “leak state”, which allows protons to flow back into the liposome lumen<sup>14</sup>.

478  
479 The further analysis of the pH traces including their fitting and determination of proton  
480 pumping/leaking rates can be done using a script we have published previously<sup>14,22</sup> and can be  
481 obtained from the Research Data Leeds Repository<sup>23,24</sup>.

482

483 **FIGURE AND TABLE LEGENDS:**

484 **Figure 1: Principle of the method.** (A) Principle of single enzyme activity monitoring and (B) the  
485 scheme of the experimental setup used in this work with a cutaway to demonstrate an internal  
486 part of the cell.

487  
488 **Figure 2: Electrochemical response of liposomes.** (A) Cole-Cole plots measured at OCP (0.22 V vs  
489 SHE; black squared line) before and after liposomes (1.3% w/w cytochrome  $bo_3$ ) adsorption from  
490 solutions at concentrations of 5  $\mu\text{g}/\text{mL}$  (red circle line) and 500  $\mu\text{g}/\text{mL}$  (blue circle line). (B) Cyclic  
491 voltammograms at 100 mV/s (left panel) and 10 mV/s (right panel) of Au-SAM (black dashed line)  
492 before and after liposomes (1.3% w/w cytochrome  $bo_3$ ) adsorption from solutions at  
493 concentrations of 5  $\mu\text{g}/\text{mL}$  (red line) and 500  $\mu\text{g}/\text{mL}$  (blue line).

494  
495 **Figure 3: Fluorescence microscopy of liposomes.** Fluorescence images of liposomes at different  
496 surface coverages: surfaces incubated for 30 min with a liposomes solution of 5  $\mu\text{g}/\text{mL}$  (top row),  
497 20  $\mu\text{g}/\text{mL}$  (middle row) and 500  $\mu\text{g}/\text{mL}$  (bottom row). Left column corresponds to 470/535 nm  
498 excitation/emission filter setup (1<sup>st</sup> HPTS channel); middle column – 410/535 nm (2<sup>nd</sup> HPTS  
499 channel); right column – 560/645 nm (FDLL channel). The images were taken at magnification  
500 90X (60X objective and 1.5X magnification by the microscope prior to the camera), 1 s exposure  
501 and similar light intensity. Scale bars correspond to 50  $\mu\text{m}$ .

502  
503 **Figure 4: Liposome identification and pH change.** (A) 3D-view of a part of fluorescence image  
504 containing a typical single liposome. Its fluorescence intensity is seen as a point spread function  
505 (color surface) that is fitted to a 2D-Gaussian function (black mesh). (B-D) pH, displayed as the  
506 median of all vesicles within single image area (typically several hundred) at different applied  
507 potentials. From 0-60 s, no potential is applied (OCP). Between 60 and 180 s (grey zone) different  
508 potentials were applied: 0 V (black), -0.1 V (red), -0.2 V (green), -0.3 V (blue). Between 180 and  
509 300 s, 0.4 V vs. SHE was applied. The cytochrome  $bo_3$  to lipid ratios were (B) 1.3%, (C) 0.1%, (D)  
510 0%. The traces are offset for clarity.

511  
512 **Figure 5: pH traces of liposomes containing 1.3% of enzyme.** Traces of pH change of 72 single  
513 liposomes, randomly selected, containing cytochrome  $bo_3$  (1.3% w/w) measured and analyzed  
514 during one experiment. From 0-60 s, no potential is applied (OCP); between 60 and 180 s (grey  
515 zone) -0.2 V vs. SHE; between 180 and 300 s, 0.4 V vs. SHE was applied.

516  
517 **Figure 6: pH traces of liposomes containing 0.1% of enzyme.** Traces of pH change of 72 single  
518 liposomes, randomly selected, containing cytochrome  $bo_3$  (0.1% w/w) measured and analyzed  
519 during one experiment. From 0-60 s, no potential is applied (OCP); between 60 and 180 s (grey  
520 zone) -0.2 V vs. SHE; between 180 and 300 s, 0.4 V vs. SHE was applied.

521  
522 **Figure 7: pH traces of liposomes without enzyme.** Traces of pH change of 72 single liposomes,  
523 randomly selected, without cytochrome  $bo_3$  measured and analyzed during one experiment.  
524 From 0-60 s: no potential is applied (OCP); between 60 and 180 s (grey zone) -0.2 V vs. SHE;  
525 between 180 and 300 s, 0.4 V vs. SHE was applied.

526  
527 **Movie 1: Animation of single liposome pH change.** (top panel) Change of a liposome

528 fluorescence on two HPTS channels (shown as 3D-surface plot of the corresponding area) during  
529 300 s of the experiment. The reductive potential was applied between 60 s and 180 s. (bottom  
530 panel) Corresponding plot of volumetric intensity ratio of two HPTS channels versus time. The  
531 zone of reductive potential application is shaded in grey.

532

### 533 **DISCUSSION:**

534 The method described is suitable to study proton pumping by respiratory membrane proteins  
535 that can be reconstituted into liposomes and are able to exchange electrons with the quinone  
536 pool. Proton pumping activity can be monitored at the single-enzyme level using pH-sensitive  
537 (ratiometric) dyes encapsulated in the liposome lumen (**Figure 1A**).

538

539 The method relies on the ability of ubiquinone (or other quinones), incorporated into the lipid  
540 bilayer, to exchange electrons with electrodes modified with a SAM<sup>18</sup>. The properties of the gold  
541 electrode, modified with a SAM, are very specific. Liposomes need to adsorb onto the SAM and  
542 the SAM needs to be thin enough to enable rapid electrochemical oxidation and reduction of the  
543 quinone pool in the liposomes. Importantly, the interaction between the electrode and liposome  
544 needs to be weak enough not to impair the integrity of the lipid membrane. Furthermore,  
545 depending on the details of the experimental system, it is desirable if the SAM prevents gold-  
546 catalyzed side reactions, such as oxygen reduction. Finally, the SAM needs to be stable within the  
547 potential window used. The use of ultra-flat gold electrodes modified with high-quality SAM of  
548 6MH fulfills these requirements in the case of cytochrome *bo*<sub>3</sub>. Stripping gold electrodes from  
549 atomically-flat silicon wafers creates an ultra-smooth gold surface with a near ideal SAM as  
550 indicated by the impedance spectra. Please note that we form the SAM from 6MH in water. We  
551 previously reported on SAMs with 6MH in isopropanol<sup>16</sup>, but these SAMs exhibit higher double-  
552 layer capacitance values and impedance spectra that indicate a more heterogeneous surface (*i.e.*,  
553 more defects) with a lower resistance. Furthermore, when SAMs from 6MH are prepared from a  
554 water solution, less background oxygen reduction (due to gold catalyzed oxygen reduction) is  
555 observed compared to SAMs formed from an ethanol/isopropanol solution. All these  
556 observations indicate that SAMs from 6MH in water solutions have a higher quality with less  
557 defects. Higher-quality SAMs can also be formed from thiol-compounds with longer alkane chains  
558 (*e.g.*, 1-hydroxy-decane-thiol), but the electron transfer kinetics become slower as the SAM  
559 thickness increases.

560

561 During (spectro)electrochemistry, chloride ions should be avoided in the buffer solution since  
562 they may chemisorb on the gold surface at high potentials, possibly deteriorating the SAM  
563 quality. For the same reason, a chloride-free reference electrode should be preferred.

564

565 Another important point is the background permeability of the liposomes to protons, which  
566 should be low enough to allow proton accumulation as a result of enzymatic proton translocation  
567 on the timescale of experiment. The exact lipid composition may influence this permeability. In  
568 our work, we use polar *E. coli* lipid extracts supplemented with ubiquinone-10. Proton  
569 permeability has been shown to depend strongly on the fatty-acid chain length<sup>25</sup>, although this  
570 has been contradicted in studies with more complex lipid compositions<sup>26</sup>. Interestingly,  
571 ubiquinone has recently been shown to enhance membrane stability<sup>27</sup>. Finally, residual

572 detergents from the protein reconstitution procedure can influence proton leakage and hence it  
573 is important to remove detergents as completely as possible using hydrophobic polystyrene  
574 microbeads, dilution followed by centrifugation and thorough rinsing of the electrochemical cell  
575 after the proteoliposomes are adsorbed on surface. If doubtful, liposome permeability can be  
576 verified by following the lumen pH change (via HPTS) in a response to an external pH jump<sup>28</sup>.

577  
578 Single enzyme measurement require unilamellar liposomes and smaller liposomes are expected  
579 to show faster or higher pH changes as the lumen's volume decreases. To achieve this,  
580 polystyrene microbeads are added gradually in small amounts leading to slow rate of liposomes  
581 formation. In such conditions, small liposomes of homogeneous size are formed with an average  
582 diameter of 70 nm and a polydispersity index of 0.24 in our case<sup>14</sup>. HPTS also adsorbs on the  
583 polystyrene microbeads, reducing the capacity of polystyrene microbeads to adsorb detergent.  
584 Hence, excess polystyrene microbeads should be added to compensate for its loss. Treatment  
585 with polystyrene microbeads was observed to reduce the HPTS concentration in the lipid-protein  
586 suspension by about a quarter (from 5 mM to 3 - 4 mM). However, the actual HPTS concentration  
587 in the liposome's lumen might be higher since the liposomes are formed early on the treatment  
588 with polystyrene microbeads. Instead of HPTS, other pH-sensitive dyes can be used. However, it  
589 is important that the dye is membrane-impermeable and ratiometric. The latter avoids  
590 experimental errors due to photobleaching and varying amounts of encapsulated dye.

591  
592 Simple calculations can be made to estimate whether, stochastically, most of the non-empty  
593 liposomes contain only one enzyme molecule. Assuming an average liposome diameter of 70 nm,  
594 a lipid molecular weight of 750 Da and a lipid area of 0.65 nm<sup>2</sup> gives us a liposome mass of  $5.2 \times 10^{-17}$   
595 g, *i.e.*,  $9.6 \times 10^{13}$  liposomes per preparation (5 mg of lipid). At 0.1% of cytochrome *bo*<sub>3</sub> (MW =  
596 144 kDa),  $2 \times 10^{13}$  enzyme molecules are present, corresponding to a 0.2 protein:liposome ratio.  
597 Using a Poisson distribution, one may further calculate that the probability to find one enzyme  
598 molecule (17%) per liposome is ten times higher than probability to find more than one molecule  
599 (1.7%). More precise calculations can be made if the losses of enzyme and lipids during the  
600 reconstitution determined from corresponding assays are taken into account. The latter can be  
601 estimated from a protein assay and by measuring FDLL fluorescence of prepared liposomes.

602  
603 Once the protocol is established and single enzyme traces are recorded, further modifications of  
604 the method is feasible depending on the aim. One might think about an enzyme inhibitor addition  
605 or introduction of an ionophore into the system. Care should be taken to verify that addition of  
606 solvents used to prepare ionophore or inhibitor stock do not influence the state of SAM or  
607 permeability of the liposomes.

608  
609 The technique as described here is limited to a particular group of enzymes that are both  
610 membrane proton transporters and quinone-converting. The equipment and experimental  
611 conditions as used here were optimized for the enzymatic activity of cytochrome *bo*<sub>3</sub>. Here, the  
612 time resolution is 2-3 seconds, determined by the exposure time and the time it takes for the  
613 turret to change filters. The duration of the experiment is limited by photobleaching of  
614 fluorescent dye and, thus, by the light intensity. We have previously found average proton  
615 translocation rate of cytochrome *bo*<sub>3</sub> to be  $73 \pm 2.2$  protons/s using this technique, although

616 activities down to 20 protons/s were detected. To use these techniques for enzymes with either  
617 more or less activity, image acquisition parameters need to be adapted (*i.e.*, light intensity,  
618 exposure time and duration of experiment). In the future, this method can be extended towards  
619 other electron transport driving proton pumping enzymes, an example being mitochondrial  
620 complex I. Other enzymes might require different reconstitution protocols, and this would need  
621 to be optimized for each different transporter. Proton pumps that are not quinone-converting  
622 enzymes can also be studied, *e.g.*, ATP-driven<sup>29</sup>, although in this case proton translocation cannot  
623 be triggered electrochemically, but addition of an initiator (*e.g.*, ATP) is required. In the latter  
624 case, there is no need to use a gold-modified cover slip. Moreover, provided that a suitable  
625 membrane-impermeable and ion-sensitive fluorescent dye is used, this method can be extended  
626 to other ionic pumps, *e.g.*, sodium and potassium.

627

#### 628 **ACKNOWLEDGMENTS:**

629 The authors acknowledge the BBSRC (BB/P005454/1) for financial support. NH was funded by  
630 the VILLUM Foundation Young Investigator Program.

631

#### 632 **DISCLOSURES:**

633 The authors have nothing to disclose.

634

#### 635 **REFERENCES:**

- 636 1. Claessen, V.I. *et al.* Single-Biomolecule Kinetics: The Art of Studying a Single Enzyme.  
637 *Annual Review of Analytical Chemistry*. **3** (1), 319–340,  
638 10.1146/annurev.anchem.111808.073638 (2010).
- 639 2. Rojek, M.J., Walt, D.R. Observing Single Enzyme Molecules Interconvert between Activity  
640 States upon Heating. *PLoS ONE*. **9** (1), e86224, 10.1371/journal.pone.0086224 (2014).
- 641 3. Velonia, K. *et al.* Single-Enzyme Kinetics of CALB-Catalyzed Hydrolysis. *Angewandte*  
642 *Chemie International Edition*. **44** (4), 560–564, 10.1002/anie.200460625 (2005).
- 643 4. Lu, H.P., Xun, L., Xie, X.S. Single-molecule enzymatic dynamics. *Science (New York, N.Y.)*.  
644 **282** (5395), 1877–82, 10.1126/science.282.5395.1877 (1998).
- 645 5. Engelkamp, H., Hatzakis, N.S., Hofkens, J., De Schryver, F.C., Nolte, R.J.M., Rowan, A.E. Do  
646 enzymes sleep and work? *Chemical Communications*. (9), 935–940, 10.1039/b516013h (2006).
- 647 6. Boukobza, E., Sonnenfeld, A., Haran, G. Immobilization in Surface-Tethered Lipid Vesicles  
648 as a New Tool for Single Biomolecule Spectroscopy. *The Journal of Physical Chemistry B*. **105** (48),  
649 12165–12170, 10.1021/jp012016x (2001).
- 650 7. Hsin, T.-M., Yeung, E.S. Single-Molecule Reactions in Liposomes. *Angewandte Chemie*  
651 *International Edition*. **46** (42), 8032–8035, 10.1002/anie.200702348 (2007).
- 652 8. García-Sáez, A.J., Schwille, P. Single molecule techniques for the study of membrane  
653 proteins. *Applied Microbiology and Biotechnology*. **76** (2), 257–266, 10.1007/s00253-007-1007-8  
654 (2007).
- 655 9. Onoue, Y. *et al.* A giant liposome for single-molecule observation of conformational  
656 changes in membrane proteins. *Biochimica et Biophysica Acta (BBA) - Biomembranes*. **1788** (6),  
657 1332–1340, 10.1016/J.BBAMEM.2009.01.015 (2009).
- 658 10. Jefferson, R.E., Min, D., Corin, K., Wang, J.Y., Bowie, J.U. Applications of Single-Molecule  
659 Methods to Membrane Protein Folding Studies. *Journal of Molecular Biology*. **430** (4), 424–437,



- 660 10.1016/J.JMB.2017.05.021 (2018).
- 661 11. Rigaud, J.L., Pitard, B., Levy, D. Reconstitution of membrane proteins into liposomes:  
662 application to energy-transducing membrane proteins. *BBA - Bioenergetics*. **1231** (3), 223–246,  
663 10.1016/0005-2728(95)00091-V (1995).
- 664 12. Jesorka, A., Orwar, O. Liposomes: Technologies and Analytical Applications. *Annual*  
665 *Review of Analytical Chemistry*. **1** (1), 801–832, 10.1146/annurev.anchem.1.031207.112747  
666 (2008).
- 667 13. Seddon, A.M., Curnow, P., Booth, P.J. Membrane proteins, lipids and detergents: Not just  
668 a soap opera. *Biochimica et Biophysica Acta - Biomembranes*. **1666** (1–2), 105–117,  
669 10.1016/j.bbamem.2004.04.011 (2004).
- 670 14. Li, M. *et al.* Single Enzyme Experiments Reveal a Long-Lifetime Proton Leak State in a  
671 Heme-Copper Oxidase. *Journal of the American Chemical Society*. **137** (51), 16055–16063,  
672 10.1021/jacs.5b08798 (2015).
- 673 15. Rumbley, J.N., Furlong Nickels, E., Gennis, R.B. One-step purification of histidine-tagged  
674 cytochrome bo<sub>3</sub> from *Escherichia coli* and demonstration that associated quinone is not required  
675 for the structural integrity of the oxidase. *Biochimica et Biophysica Acta (BBA) - Protein Structure*  
676 *and Molecular Enzymology*. **1340** (1), 131–142, 10.1016/S0167-4838(97)00036-8 (1997).
- 677 16. Jeuken, L.J.C. *et al.* Phase separation in mixed self-assembled monolayers and its effect  
678 on biomimetic membranes. *Sensors and Actuators, B: Chemical*. **124** (2), 501–509,  
679 10.1016/j.snb.2007.01.014 (2007).
- 680 17. Barsoukov, E., Macdonald, J.R. *Impedance Spectroscopy Theory, Experiment, and*  
681 *Applications*. Chapters 1-2. John Wiley & Sons. (2005).
- 682 18. Jeuken, L.J.C., Connell, S.D., Henderson, P.J.F., Gennis, R.B., Evans, S.D., Bushby, R.J. Redox  
683 Enzymes in Tethered Membranes. *Journal of the American Chemical Society*. **128** (5), 1711–1716,  
684 10.1021/ja056972u (2006).
- 685 19. Compton, R.G., Banks, C.E. *Understanding voltammetry*. Chapter 4. World Scientific  
686 Publishing Europe Ltd. Singapore. (2018).
- 687 20. Girault, H.H. *Analytical and Physical Electrochemistry*. Chapter 10. EPFL Press. Lausanne.  
688 (2004).
- 689 21. Mazurenko, I., Jeuken, L.J.C. Timelapse (movie) of single vesicles fluorescence change  
690 upon potential application. 10.5518/442 (2018).
- 691 22. Li, M., Khan, S., Rong, H., Tuma, R., Hatzakis, N.S., Jeuken, L.J.C. Effects of membrane  
692 curvature and pH on proton pumping activity of single cytochrome bo<sub>3</sub>enzymes. *Biochimica et*  
693 *Biophysica Acta - Bioenergetics*. **1858** (9), 763–770, 10.1016/j.bbabi.2017.06.003 (2017).
- 694 23. Li, M., Tuma, R., Jeuken, L.J.C. MATLAB code for the analysis of proton transport activity  
695 in single liposomes. 10.5518/150 (2017).
- 696 24. Li, M., Tuma, R., Jeuken, L.J.C. Time-resolved fluorescence microscopic data (traces) of  
697 individual lipid vesicles with proton transport activity. 10.5518/151 (2017).
- 698 25. Paula, S., Volkov, A.G., Van Hoek, A.N., Haines, T.H., Deamer, D.W. Permeation of protons,  
699 potassium ions, and small polar molecules through phospholipid bilayers as a function of  
700 membrane thickness. *Biophysical Journal*. **70** (1), 339–348, 10.1016/S0006-3495(96)79575-9  
701 (1996).
- 702 26. Brookes, P.S., Hulbert, A.J., Brand, M.D. The proton permeability of liposomes made from  
703 mitochondria inner membrane phospholipids: No effect of fatty acid composition. *Biochimica et*

704 *Biophysica Acta (BBA) - Biomembranes*. **1330** (2), 157–164, 10.1016/S0005-2736(97)00160-0  
705 (1997).

706 27. Eriksson, E.K., Agmo Hernández, V., Edwards, K. Effect of ubiquinone-10 on the stability  
707 of biomimetic membranes of relevance for the inner mitochondrial membrane. *Biochimica et*  
708 *Biophysica Acta - Biomembranes*. **1860** (5), 1205–1215, 10.1016/j.bbamem.2018.02.015 (2018).

709 28. Seneviratne, R. *et al.* A reconstitution method for integral membrane proteins in hybrid  
710 lipid-polymer vesicles for enhanced functional durability. *Methods*. 1–8,  
711 10.1016/j.ymeth.2018.01.021 (2018).

712 29. Veshaguri, S. *et al.* Direct observation of proton pumping by a eukaryote P-type ATPase.  
713 *Science*. **351** (6280), 1–6 (2016).

714