SUPPLEMENTAL MATERIAL FOR

Positive Feedback Amplifies the Response of Mitochondrial Membrane Potential to Glucose Concentration in Clonal Pancreatic Beta Cells

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Appendix A **Potentiometric calibration equations and parameters**

Time courses of TMRM and PMPI fluorescence intensities were converted to millivolts using previously derived [22] (see references in main text) calibration equations (Eq. 1-Eq. 2) and parameters given in text and in Suppl. Table 2. Notably, an absolute value calculation in the argument of the logarithm was included in Eq. 2 to prevent data clipping at discharged ΔψM where noise in the recording may render the argument occasionally negative. For each probe (indices starting with P are for PMPI and T for TMRM) these equations calculate the potentials for each time point that result in an equilibrium fluorescence (***F***) when the temporal derivative (***D***) of the fluorescence time course is zero, or in disequilibrium (***F*** is changing in time, thus ***D***≠0). ***F*** is normalized relative to baseline of 1. ***D*** was calculated from ***F*** by Savitzly-Golay kernel differentiation (see kernel parameters in Suppl. Table 2).

$$\Delta\Psi_P = -2\frac{RT}{z_P F}\ln\frac{D_P + \sqrt{{D_P}^2 + 4(F_P - f_{PX})(f_{P0} - f_{PX}){k_P}^2}}{2(f_{P0} - f_{PX})k_P} \qquad \text{Eq. 1}$$

$$\Delta\Psi_M = -\frac{RT}{z_{TM}F}\ln\left[\left|\frac{1}{R_{AV}} - 1\left(\frac{k_T R_{AV} E(F_T - f_{TX})}{k_T(f_{T0} - f_{TX}) - D_T R_{AV} E^{s_T}} - 1\right)\right|\right] \qquad \text{Eq. 2}$$

where $E = e^{\frac{z_T F}{RT}\Delta\Psi_P(t)}$ and $R_{AV} = \frac{1 + V_F(1/a'_R - 1)}{1 - V_F V_{FM}}$

To calculate millivolts, first all calibration parameters in (Eq. 1-Eq. 2) need to be obtained. We have previously designed and used an internal calibration paradigm [16,22,25], where at the end of each time course calibrants were added to the sample allowing calculation of the parameters

for the given recording. In the current work we describe how to obtain the calibration parameters in a simplified and scalable experimental design applicable to certain fluorescence microplate readers.

Calibration parameters include of a set of physical and biophysical constants: ***R*** is the molar gas constant (8.314 $J{\cdot}K^{-1}{\cdot}mol^{-1}$), ***T*** is the temperature (310 K), ***F*** is the Faraday constant (96485 $C{\cdot}mol^{-1}$), ***z*** is the signed apparent charge of the probes determined in [22]; TMRM at the plasma membrane (z_T=0.80) at the mitochondrial inner membrane (z_{TM}=0.71) and of PMPI at the plasma membrane (z_P=-0.55).

Specimen-specific parameters are the mitochondria:cell volume fraction (V_F) and the apparent activity coefficient ratio (a_R', which expresses ultrastructural parameters, differences in chemical activity between the mitochondrial matrix and the cytosol, and optical dilution). The R_{AV} term in Eq. 2 consolidates these geometric and affinity terms into a single parameter. While the matrix:mitochondria volume fraction (V_{FM}) is an explicit parameter here, the measurement of a_R' largely accounts for TMRM accumulation in the matrix volume and not in the entire mitochondrion. This is because the fractional occupancy of the mitochondrial volume by the matrix results in optical dilution of TMRM fluorescence that is reflected by the measured a_R'. The calculated ΔψM is therefore insensitive to V_{FM}, and a fixed 0.63 value previously measured in INS-1E cells was used here [22].

Lastly, a recording-specific set of parameters comprise the potential-independent background fluorescence that can be calculated but not directly measured (f_{PX} and f_{TX}; including autofluorescence and probe binding), the directly measured probe fluorescence at 0 mV potential (f_{P0} and f_{T0}) and the rate constant of probe uptake at zero potential (k_P and k_T).

Appendix B **Linear spectral unmixing**

$$M = \begin{bmatrix} 1 & \frac{\mathrm{TMRM}_{\mathrm{PMPI}}}{\mathrm{PMPI}_{\mathrm{PMPI}}} \\ \frac{\mathrm{PMPI}_{\mathrm{TMRM}}}{\mathrm{TMRM}_{\mathrm{TMRM}}} & 1 \end{bmatrix}^{-1} \qquad \text{Eq. 3}$$

$$\begin{bmatrix} \mathrm{TMRM}_{\mathrm{Unmixed}} \\ \mathrm{PMPI}_{\mathrm{Unmixed}} \end{bmatrix} = M . \begin{bmatrix} \mathrm{TMRM} \\ \mathrm{PMPI} \end{bmatrix} \qquad \text{Eq. 4}$$

Fluorescence crossbleed between TMRM and PMPI was corrected by linear spectral unmixing. A 2×2 transformation matrix (***M***) was calculated from the intensities measured in the two (TMRM and PMPI) fluorescence channels in wells with a cell monolayer stained either with TMRM or PMPI (indices in Eq. 3). The PMPI wells were treated with CDC to increase fluorescence. Corresponding cell-free blank well fluorescence (with matching medium) was subtracted before calculation of ***M***. ***M*** was then refined for every experiment using numerical optimization in Mathematica to adjust the top right coefficient in Eq. 3 to give zero cross-correlation coefficient calculated between unmixed TMRM and PMPI fluorescence time courses after CDC addition. In this condition PMPI fluorescence always gradually increases while a change in TMRM fluorescence is no longer resolvable, based on fluorescence microscopic observations. Finally, pairs of spectrally unmixed fluorescence intensities were calculated for each measurement well using Eq. 4 after matched blank well background subtraction. The values obtained were used for the potentiometric calibration below.

Appendix C **Calculation of $\Delta\psi P_0$, $\Delta\psi M_0$ and k_T**

$\Delta\psi M_0$ and k_T were determined using the TMRM fluorescence decay method described in [22], with modifications to allow for an unsteady baseline (non-zero first temporal derivative of TMRM fluorescence at baseline; $\boldsymbol{D_{T0}}$). $\Delta\psi P_0$ was determined from the same data, by numerical optimization of the $\Delta\psi M$ calibration below seeking a value of $\Delta\psi P_0$ at which $\Delta\psi M$ calibration has the minimal deviation from zero potential during application of CDC, where $\Delta\psi P$ is gradually approaching to zero. In four microplate columns (16 wells as technical replicates and 16 corresponding blank wells), after recording baseline, $\Delta\psi M$ was discharged by MDC, then $\Delta\psi P$ was also discharged by CDC and equilibrium TMRM fluorescence corresponding to 0 mV potentials (f_{T0}) was measured. During numerical optimization of $\Delta\psi P_0$, first $\Delta\psi P$ was calculated for each time point as given in Appendix E using a two-point calibration between the test value for $\Delta\psi P_0$ and 0 mV. Then $\Delta\psi M_0$ was calculated by Eq. 5 where *slope* and *intercept* refer to parameters of the linear regression on the data points obtained by plotting $D_{TE} = D_T E^{S_T} / (E-1)$ as a function of $F_{TE} = (f_{T0} - F_T E) / (E-1)$ for each time point following MDC addition (see Fig. 2D), where $E = e^{\frac{z_T F}{RT}\Delta\Psi_P(t)}$ and $\boldsymbol{F_T}$ and $\boldsymbol{D_T}$ are TMRM fluorescence and its first temporal derivative, respectively. The median of the parameters determined in the 16 wells with cells was used for further calculations.

$$\Delta\Psi_{M0} = -b_{TM} \ln\left[\frac{1}{R_{AV}-1}\left(\frac{R_{AV}E_0(1-f_{TX})k_T}{(f_{T0}-f_{TX})k_T - E_0^{\,S_T} R_{AV} D_{T0}} - 1\right)\right] \qquad \text{Eq. 5}$$

where $k_T = slope \cdot R_{AV}$, $f_{TX} = intercept / slope$, $E_0 = e^{\frac{z_T F}{RT}\Delta\Psi_{P0}}$.

Appendix D **Quality control**

To obtain $\Delta\psi M_0$, the longest and most linear section of the $\boldsymbol{D_{TE}}$ vs $\boldsymbol{F_{TE}}$ graph (defined in Appendix C and shown in Fig. 2D) was used for linear regression automatically, defined by running differentiation and the median of the derivatives for the ($\boldsymbol{D_{TE}}$, $\boldsymbol{F_{TE}}$) data points. Wells with r^2 of the linear regression less than 0.5 were discarded. $\Delta\psi M_0$ was not calculated in wells where the absolute value of mean $\Delta\psi M$, or its SD was larger than 25 mV following MDC addition. $\Delta\psi M$ data points outside the range -250 to 50 mV were discarded both for baseline potential determinations and the "short" calibration paradigm below.

Determinations of respiration rate were quality controlled by a requirement for glucose activation of baseline respiration.

Appendix E **Baseline to zero ("short") potentiometric calibration**

To calibrate an arbitrary recording (e.g. rotenone or FCCP titrations) a "short" (baseline to zero) calibration paradigm was used, relying on two calibration points; the baseline (which may be in disequilibrium for TMRM) and complete ($\Delta\psi P$ and $\Delta\psi M$) depolarization. To this end we used the values of $\Delta\psi P_0$, $\Delta\psi M_0$ and k_T measured above, which are assumed to be sample/condition specific, but independent of the amount of specimen in a well, and the $\boldsymbol{f_{T0}}$ and $\boldsymbol{f_{P0}}$ values measured for each well after complete depolarization of both potentials. Eq. 6-Eq. 7 were derived by solving

the main calibration equations (Eq. 1 and Eq. 2) for f_{PX} and f_{TX}, respectively, at $\Delta\psi = \Delta\psi_0$ and F=1. Finally, with all calibration parameters known, Eq. 1 and Eq. 2 were used to calculate potentials.

$$f_{PX} = 1 - \frac{f_{P0} - 1}{e^{\frac{z_P F}{\mathrm{RT}}\Delta\psi \mathrm{P0}} - 1} \qquad \text{Eq. 6}$$

$$f_{\mathrm{TX}} = 1 - \frac{(-1 + e^{\frac{F\Delta\psi \mathrm{M0} z_{\mathrm{TM}}}{RT}} + R_{\mathrm{AV}})(-(-1 + f_{\mathrm{T0}})k_T + (e^{\frac{F\Delta\psi \mathrm{P0} z_T}{RT}})^{s_T} D_{\mathrm{T0}} R_{\mathrm{AV}})}{k_T(-1 + e^{\frac{F\Delta\psi \mathrm{M0} z_{\mathrm{TM}}}{RT}} - (-1 + e^{\frac{F(\Delta\psi \mathrm{P0} z_T + \Delta\psi \mathrm{M0} z_{\mathrm{TM}})}{RT}})R_{\mathrm{AV}})} \qquad \text{Eq. 7}$$

Appendix F **Modular Control and Regulation Analysis**

Elasticities and control coefficients were calculated as in [17]. Response coefficients were calculated as in [18,30]. However, for the sake of clarity, we provide these equations as we used them in Mathematica (Eq. 11 - Eq. 14).

$$\mathrm{N} = [1 \quad -1 \quad -1] \qquad \text{Eq. 8}$$

$$\mathrm{dJ}^0 = \begin{bmatrix} J_O & 0 & 0 \\ 0 & J_P & 0 \\ 0 & 0 & J_L \end{bmatrix} \qquad \text{Eq. 9}$$

$$\varepsilon = \begin{bmatrix} \varepsilon^{\mathrm{O}}_{\Delta\mathrm{p}} \\ \varepsilon^{\mathrm{P}}_{\Delta\mathrm{p}} \\ \varepsilon^{\mathrm{L}}_{\Delta\mathrm{p}} \end{bmatrix} \qquad \text{Eq. 10}$$

$$^{*}C_i^{\Delta\psi M} = -(N \cdot \mathrm{dJ}^0 \cdot \varepsilon)^{-1} \cdot N \cdot \mathrm{dJ}^0 = \\ = \left[\frac{J_O}{\varepsilon^{\mathrm{L}}_{\Delta\mathrm{p}} J_L - \varepsilon^{\mathrm{O}}_{\Delta\mathrm{p}} J_O + \varepsilon^{\mathrm{P}}_{\Delta\mathrm{p}} J_P} \quad -\frac{J_P}{\varepsilon^{\mathrm{L}}_{\Delta\mathrm{p}} J_L - \varepsilon^{\mathrm{O}}_{\Delta\mathrm{p}} J_O + \varepsilon^{\mathrm{P}}_{\Delta\mathrm{p}} J_P} \quad -\frac{J_L}{\varepsilon^{\mathrm{L}}_{\Delta\mathrm{p}} J_L - \varepsilon^{\mathrm{O}}_{\Delta\mathrm{p}} J_O + \varepsilon^{\mathrm{P}}_{\Delta\mathrm{p}} J_P} \right] \qquad \text{Eq. 11}$$

where $^{*}C_i^{\Delta\psi M}$ is an i-dimensional row vector or its elements at i =(O,P,L) in this order,

$$^{*}C_i^{J_j} = I_i + \varepsilon \cdot {}^{*}C_i^{\Delta\psi M} \\ = \begin{bmatrix} 1 + \frac{\varepsilon^{\mathrm{O}}_{\Delta\mathrm{p}} J_O}{\varepsilon^{\mathrm{L}}_{\Delta\mathrm{p}} J_L - \varepsilon^{\mathrm{O}}_{\Delta\mathrm{p}} J_O + \varepsilon^{\mathrm{P}}_{\Delta\mathrm{p}} J_P} & -\frac{\varepsilon^{\mathrm{O}}_{\Delta\mathrm{p}} J_P}{\varepsilon^{\mathrm{L}}_{\Delta\mathrm{p}} J_L - \varepsilon^{\mathrm{O}}_{\Delta\mathrm{p}} J_O + \varepsilon^{\mathrm{P}}_{\Delta\mathrm{p}} J_P} & -\frac{\varepsilon^{\mathrm{O}}_{\Delta\mathrm{p}} J_L}{\varepsilon^{\mathrm{L}}_{\Delta\mathrm{p}} J_L - \varepsilon^{\mathrm{O}}_{\Delta\mathrm{p}} J_O + \varepsilon^{\mathrm{P}}_{\Delta\mathrm{p}} J_P} \\ \frac{\varepsilon^{\mathrm{P}}_{\Delta\mathrm{p}} J_O}{\varepsilon^{\mathrm{L}}_{\Delta\mathrm{p}} J_L - \varepsilon^{\mathrm{O}}_{\Delta\mathrm{p}} J_O + \varepsilon^{\mathrm{P}}_{\Delta\mathrm{p}} J_P} & 1 - \frac{\varepsilon^{\mathrm{P}}_{\Delta\mathrm{p}} J_P}{\varepsilon^{\mathrm{L}}_{\Delta\mathrm{p}} J_L - \varepsilon^{\mathrm{O}}_{\Delta\mathrm{p}} J_O + \varepsilon^{\mathrm{P}}_{\Delta\mathrm{p}} J_P} & -\frac{\varepsilon^{\mathrm{P}}_{\Delta\mathrm{p}} J_L}{\varepsilon^{\mathrm{L}}_{\Delta\mathrm{p}} J_L - \varepsilon^{\mathrm{O}}_{\Delta\mathrm{p}} J_O + \varepsilon^{\mathrm{P}}_{\Delta\mathrm{p}} J_P} \\ \frac{\varepsilon^{\mathrm{L}}_{\Delta\mathrm{p}} J_O}{\varepsilon^{\mathrm{L}}_{\Delta\mathrm{p}} J_L - \varepsilon^{\mathrm{O}}_{\Delta\mathrm{p}} J_O + \varepsilon^{\mathrm{P}}_{\Delta\mathrm{p}} J_P} & -\frac{\varepsilon^{\mathrm{L}}_{\Delta\mathrm{p}} J_P}{\varepsilon^{\mathrm{L}}_{\Delta\mathrm{p}} J_L - \varepsilon^{\mathrm{O}}_{\Delta\mathrm{p}} J_O + \varepsilon^{\mathrm{P}}_{\Delta\mathrm{p}} J_P} & 1 - \frac{\varepsilon^{\mathrm{L}}_{\Delta\mathrm{p}} J_L}{\varepsilon^{\mathrm{L}}_{\Delta\mathrm{p}} J_L - \varepsilon^{\mathrm{O}}_{\Delta\mathrm{p}} J_O + \varepsilon^{\mathrm{P}}_{\Delta\mathrm{p}} J_P} \end{bmatrix} \qquad \text{Eq. 12}$$

where $^{*}C_i^{J_j}$ is a j-row i-column matrix, or its elements at i =(O,P,L) and j =(O,P,L) in this order.

$$ {}^{i}R^{\Delta\psi M}_{\Delta\psi M} = diag\left({}^{*}C_i^{\Delta\psi M} \right) \cdot \varepsilon = \begin{bmatrix} \dfrac{\varepsilon^{\mathrm{O}}_{\Delta\mathrm{p}} J_O}{\varepsilon^{\mathrm{L}}_{\Delta\mathrm{p}} J_L - \varepsilon^{\mathrm{O}}_{\Delta\mathrm{p}} J_O + \varepsilon^{\mathrm{P}}_{\Delta\mathrm{p}} J_P} \\ -\dfrac{\varepsilon^{\mathrm{P}}_{\Delta\mathrm{p}} J_P}{\varepsilon^{\mathrm{L}}_{\Delta\mathrm{p}} J_L - \varepsilon^{\mathrm{O}}_{\Delta\mathrm{p}} J_O + \varepsilon^{\mathrm{P}}_{\Delta\mathrm{p}} J_P} \\ -\dfrac{\varepsilon^{\mathrm{L}}_{\Delta\mathrm{p}} J_L}{\varepsilon^{\mathrm{L}}_{\Delta\mathrm{p}} J_L - \varepsilon^{\mathrm{O}}_{\Delta\mathrm{p}} J_O + \varepsilon^{\mathrm{P}}_{\Delta\mathrm{p}} J_P} \end{bmatrix} \qquad \text{Eq. 13} $$

where ${}^{i}R^{\Delta\psi M}_{\Delta\psi M}$ is a column vector or its elements at i =(O,P,L) in this order.

$$ {}^{i}IR^{\Delta\psi M}_{\Delta glucose} = diag\left({}^{*}C_i^{\Delta\psi M} \right) \cdot (\Delta \mathrm{J} - \varepsilon \cdot \Delta \mathrm{X}) = \begin{bmatrix} \dfrac{-\mathrm{J_O}\varepsilon^{\mathrm{O}}_{\Delta\mathrm{p}}\mathrm{x'} + (\mathrm{J'_O} + \mathrm{J_O}(-1 + \varepsilon^{\mathrm{O}}_{\Delta\mathrm{p}}))\mathrm{x}}{(\mathrm{J_L}\varepsilon^{\mathrm{L}}_{\Delta\mathrm{p}} - \mathrm{J_O}\varepsilon^{\mathrm{O}}_{\Delta\mathrm{p}} + \mathrm{J_P}\varepsilon^{\mathrm{P}}_{\Delta\mathrm{p}})\mathrm{x}} \\ \dfrac{\mathrm{J_P}\varepsilon^{\mathrm{P}}_{\Delta\mathrm{p}}\mathrm{x'} - (\mathrm{J'_P} + \mathrm{J_P}(-1 + \varepsilon^{\mathrm{P}}_{\Delta\mathrm{p}}))\mathrm{x}}{(\mathrm{J_L}\varepsilon^{\mathrm{L}}_{\Delta\mathrm{p}} - \mathrm{J_O}\varepsilon^{\mathrm{O}}_{\Delta\mathrm{p}} + \mathrm{J_P}\varepsilon^{\mathrm{P}}_{\Delta\mathrm{p}})\mathrm{x}} \\ \dfrac{\mathrm{J_L}\varepsilon^{\mathrm{L}}_{\Delta\mathrm{p}}\mathrm{x'} - (\mathrm{J'_L} + \mathrm{J_L}(-1 + \varepsilon^{\mathrm{L}}_{\Delta\mathrm{p}}))\mathrm{x}}{(\mathrm{J_L}\varepsilon^{\mathrm{L}}_{\Delta\mathrm{p}} - \mathrm{J_O}\varepsilon^{\mathrm{O}}_{\Delta\mathrm{p}} + \mathrm{J_P}\varepsilon^{\mathrm{P}}_{\Delta\mathrm{p}})\mathrm{x}} \end{bmatrix} \qquad \text{Eq. 14} $$

where ${}^{i}IR^{\Delta\psi M}_{\Delta glucose}$ is an i-dimensional column vector or its elements at i =(O,P,L) in this order, and

$\Delta \mathrm{J} = \begin{bmatrix} \frac{-\mathrm{J_O}+\mathrm{J'_O}}{\mathrm{J_O}} \\ \frac{-\mathrm{J_P}+\mathrm{J'_P}}{\mathrm{J_P}} \\ \frac{-\mathrm{J_L}+\mathrm{J'_L}}{\mathrm{J_L}} \end{bmatrix}$ and $\Delta \mathrm{X} = \begin{bmatrix} \frac{\mathrm{x'}-\mathrm{x}}{\mathrm{x}} \end{bmatrix}$.

Notations follow as given in [30], with modifications. For a system with i=3 modules (i=O,P,L) linked by m=1 metabolite (ΔψM), N is the m-row, i-column matrix of reaction stoichiometries, dJ^0 is a diagonal matrix of i steady state fluxes and ε is the i-row, m-column matrix of elasticities. I_i stands for an $i \times i$ identity matrix and diag() for conversion of a vector to diagonal matrix. $\mathrm{J_i}$ and x are steady state fluxes and ΔψM, while $\mathrm{J'_i}$ and $\mathrm{x'}$ are their steady state values after perturbation.

Weighed linear fits were obtained by using the LinearModelFit standard function of Mathematica with Weights -> $1/SE^2$ and VarianceEstimatorFunction -> (1 &) options. To account for measurement errors along both axes, the fit was iteratively performed using $SE^2 = slope^2 * SE_x^2 + SE_y^2$.

Error propagation was performed by calculating derivatives of Eq. 11-Eq. 14 for each measured variable or elasticity and then calculating the square root of the square sum of the product of the respective derivatives and standard errors.

Suppl. Table 1 **Calibration cocktail compositions**. Compositions follow [22], with the mitochondrial depolarization cocktail here including the components originally separately defined as “anti swelling cocktail”.

Mitochondrial depolarization cocktail (MDC) three components: 1:1000 in EtOH, 1:1000 in DMSO and 1:1000 in H_2O		
Compound	**EtOH stock (mM)**	**Final (µM)**
valinomycin	10	1
oligomycin	10	2
antimycin	20	2
FCCP	10	1
Compound	**DMSO stock (mM)**	**Final (µM)**
IAA-94	1000	100
DIOA	100	10
bumetanide	200	80
Compound	**Aqueous stock (mM)**	**Final (µM)**
tetrodotoxin	1	1
Complete depolarization cocktail (CDC) 1:500 in EtOH		
Compound	**EtOH stock (mM)**	**Final (µM)**
valinomycin	10	1
gramicidin	20	10
nigericin	100	10
monensin	100	10
FCCP	10	1
oligomycin	10	2
iodoacetate	2000	500
cyclosporin A	10	2
antimycin A	20	2

Suppl. Table 2 **Potentiometric calibration parameters.** Nomenclature follows definitions given in [22] and in the “Membrane Potential Calibration Wizard” in Image Analyst MKII.

Parameter	**Value**
a_R'	0.36±0.05
V_F	6.3±0.49%
V_{FM}	63%
k_P	0.38
Determination of $\Delta\psi M_0$ and $\Delta\psi M_0$	
Acquisition interval	19 s
Differentiation kernel for PMPI	Width=15, polynomial order=3
Differentiation kernel for TMRM	Width=15, polynomial order=2
Baseline to Zero “Short” Calibration	
Acquisition interval	36 s
Differentiation kernel for PMPI	Width=11, polynomial order=3
Differentiation kernel for TMRM	Width=11, polynomial order=2
Microscopy	
Acquisition interval	~ 60 s
Differentiation kernel for PMPI	Width=7, polynomial order=3
Differentiation kernel for TMRM	Width=7, polynomial order=2
P_N	0