

# Supplements

## Confirming the validity of responsiveness to glucose as a marker for $\beta$ -cells

Glucose-induced  $\Delta\Psi$  hyperpolarization was used as a  $\beta$ -cell marker. This criterion was validated as follows. To perform sequential imaging of living as well as fixed cells we used the Optical LiveCell array (Molecular Cytomics, Boston, MA), a honeycomb shaped cell retainer (1). Dispersed cells were plated on PDL coated arrays on glass slide bottom dishes, cultured for 2 days and stained with 7 nM TMRE. The same cells were imaged at 3 mM followed by 8 mM glucose by confocal microscopy. Next, cells were washed with PBS, fixed with 4 % paraformaldehyde (Boston BioProducts, Worcester, MA) for 15 min, permeabilized with 0.2 % Triton X-100 (Sigma-Aldrich, St Louis, MO) for 10 min and incubated with 1 % bovine serum albumin (Calbiochem, LaJolla, CA) in PBS for 15 min to reduce non-specific antibody (ab) binding, all at room temperature. A guinea pig polyclonal ab to insulin (1:100 dilution, Abcam, Cambridge, MA) was then applied for 30 min, followed by washing and incubation for 30 min with Alexa fluor 488 goat anti-guinea pig IgG ab (1:500 dilution, Invitrogen, Carlsbad, CA). The same cells were then imaged for insulin staining, now excited with a 488 nm argon laser. This protocol allowed a large number of individual cells to be monitored through several imaging steps. Using a threshold of 8 % increase, or  $\sim 2$  mV, TMRE fluorescence intensity increase (FI) was matched to positive insulin staining, revealing that 93 % of the cells responding to glucose were  $\beta$ -cells ( $n = 534$ , Fig. S1). FI change of individual cells in Fig. S1 are shown in Table 1. The mean FI change of the non  $\beta$ -cells was estimated to -1.4 %. While  $\alpha$ -cells and other islet cells are also under glucose control,  $\alpha$ -cell mitochondria are not thought to respond metabolically to ambient glucose concentrations (9).  $\beta$ -cell mitochondria are thus thought to constitute the majority of glucose-responsive pancreatic mitochondria. HEK 293 cells stained as described above and islet cells stained only with the secondary ab showed minimal fluorescence, demonstrating the protocol's specificity for insulin.

## Details of $\Delta\Psi$ analysis

Since changes in the TMRE signal can originate from changes in  $\Delta\Psi$  or can be due to focal plane shift, we used the ratio in fluorescence intensity of TMRE and MTG or PA-GFP<sub>mt</sub>. As mitochondrial movement affects both TMRE and MTG signals similarly, the ratio intensity is movement-independent (Fig. 1D). Additionally, as MTG is unaffected by  $\Delta\Psi$  (Fig. 1B-C), the TMRE/MTG ratio reflects the  $\Delta\Psi$ .

With the kind help of Molecular Devices (Sunnyvale, CA), a MetaMorph software algorithm for image analysis was constructed as follows. The analysis algorithm first extracted green areas (MTG) larger than 11 pixels that met a defined intensity threshold. In this way unspecific background staining was excluded. Dividing the red (TMRE) by green signals from these selected areas generated a ratio image.  $\Delta\Psi$  was calculated as follows.

According to the Nernst equation, partitioning of fluorescent molecules across the mitochondrial membranes can be used for determining the potential across it (2, 3).

$$\Delta\Psi = RT \cdot \log(FI_m/FI_c)$$

where  $RT = 61.5$  at  $37^\circ\text{C}$ ,  $FI_m$  = Fluorescence Intensity of mitochondria,  $FI_c$  = FI of cytosol or nucleus. As described by O'Reilly et al. (2), the  $\Delta\Psi$ -dependent component of TMRE will accumulate in a Nernstian fashion that can be described by the intensity of its fluorescence.

The non  $\Delta\Psi$ -dependent component of TMRE, also known as the binding component, can be ignored when calculating relative changes in  $\Delta\Psi$  as it is fixed and voltage-independent (2). The relative difference in  $\Delta\Psi$  under two different conditions (e.g. change in fuel exposure; Fig. 2D) is derived as follows.

$$\begin{aligned}\Delta\Psi &= RT*\log(FI_{mA}/FI_{cA}) - 61.5\log(FI_{mB}/FI_{cB}) \\ &= RT*\log[(FI_{mA}/FI_{cA})*(FI_{cB}/FI_{mB})] \\ &= RT*\log[(FI_{mA}/FI_{mB})*(FI_{cB}/FI_{cA})] \\ FI_{mA}/FI_{mB} &= (FI_{mA_{TMRE}}/FI_{mA_{MTG}})/(FI_{mB_{TMRE}}/FI_{mB_{MTG}}) = Ratio_A/Ratio_B \\ (1) \quad \Delta\Psi &= RT*\log[(Ratio_A/Ratio_B)*(FI_{cB}/FI_{cA})]\end{aligned}$$

where  $FI_{mA}$  = FI of mitochondria condition A (TMRE/MTG),  $FI_{mB}$  = FI of mitochondria condition B (TMRE/MTG),  $FI_{cA}$  = FI of cytosol or nucleus condition A (TMRE),  $FI_{cB}$  = FI of cytosol or nucleus condition B (TMRE),  $Ratio_A$  = ratio TMRE/MTG condition A and  $Ratio_B$  = ratio TMRE/MTG condition B. The FI of “cytosolic” TMRE was measured either in the nucleus or cytosol, as it has previously been demonstrated that the TMRE concentrations in these compartments are equivalent (4).

To measure the  $\Delta\Psi$  of a single mitochondrion as a function of the average of the entire focal section’s mitochondrial population (Fig. 2A), we applied the following equation.

$$(2) \quad \Delta\Psi = RT*\log(FI_{sm}/FI_{es})$$

where  $FI_{sm}$  = ratio FI of a single mitochondrion and  $FI_{es}$  = ratio FI of the entire section.

In order to assess the frequency of  $\Delta\Psi$  values and thereby the distribution range of  $\Delta\Psi$ , the ratio FI of each pixel was plotted in a histogram. By applying the equation

$$(3) \quad \Delta\Psi = RT*\log(FI_{sp}/FI_{es})$$

where  $FI_{sp}$  = ratio FI of a single pixel, FI was converted to mV (Fig. 2B-C, 4).

The relative disparity in  $\Delta\Psi$  between two individual mitochondria (Fig. 3B) was determined by the application of the equation

$$(4) \quad \Delta\Psi = RT*\log(R_{m1}/R_{m2})$$

where  $R_{m1}$  = Ratio TMRE/GFP of mitochondrion 1 and  $R_{m2}$  = Ratio TMRE/GFP of mitochondrion 2

### **Contribution of noise to heterogeneity**

The heterogeneity of  $\Delta\Psi$  may possibly in part be explained by noise. In order to give a quantitative estimation of the contribution of non-biologic influences, e.g. light scattering and microscope detector noise, on the measurements of heterogeneity the following components have been studied. Taken together, the overall noise due to imaging procedures is estimated to contribute a maximum of 1.6 SD per mitochondrion which would translate into 1.97 SD for an imaging section with ~100 mitochondria.

*Variability generated through damage introduced during cellular preparation or dye distribution:* Cells under stress, e.g. may react with mitochondrial swelling (5). This might in theory affect the measurement of  $\Delta\Psi$  heterogeneity and create artefacts. To test for this, we measured mitochondrial cross diameter in whole islet cells and dispersed islet cells, all stained with 13  $\mu\text{M}$  of the  $\Delta\Psi$  sensitive dye Rho123 for 30 min ( $n = 147$  and 125 mitochondria respectively). There was no difference in diameter between the two groups of mitochondria (Fig. S2A). Mitochondrial diameter was also compared between cells incubated under GLT conditions and control cells ( $n = 84$  and 92 mitochondria respectively, all stained with TMRE and MTG (Fig. S2B). There was no significant difference in mitochondrial diameter between the two groups of cells, which is similar to previously reported in HeLa cells (10). Further, to exclude artefacts due to primary cell culture, heterogeneity in  $\Delta\Psi$  was tested in the  $\beta$ -cell line INS1. Fig. S2C show an example of an INS1 cell, stained with MTG and TMRE (here in pseudo-colour). It is apparent that the heterogeneity in  $\Delta\Psi$  shown in Fig. 2A, also exists in INS1 cells. Finally, TMRE may become diluted in damaged mitochondria that go through swelling even if actual membrane potential does not drop. To rule out such false positive interpretations of  $\Delta\Psi$  depolarization in large mitochondria, the correlation between mitochondrial size and  $\Delta\Psi$  was studied (Fig. S2D). Depolarized mitochondria were found to be smaller than the average, which is expected since fission is triggered by depolarisation.

#### *Noise of the methodology*

*Stability of the heterogeneity parameter measured from the same cell over time:* Time laps measurements of  $\Delta\Psi$  heterogeneity (SD) were performed in order to determine to what extent noise inserted by the imaging acquisition, processing and analysis procedures alter the heterogeneity value. Analysis of consecutive images obtained from the same cell, with an interval of 23 s, revealed a variability in the heterogeneity value which was 2 % ( $n = 10$  cells, Fig. S3A).

*Spatial variability in the imaging field:* 10.2  $\mu\text{m}$  fluorescent beads (Bangs Laboratories, Fishers, IN) were embedded in 1 % agarose gel on a glass slide bottom dish. Confocal microscopy images were recorded using 488 and 561 excitation lasers and analyzed similar to the live cells (Fig. S3B). The ratio of green and red fluorescence light was quantified and the same mathematical approach used to calculate  $\Delta\Psi$  differences was applied. Differences in FI measured from the different beads were maximally 4 %, which would be equivalent to values of 1 mV, if these were measurements obtained from mitochondria stained with TMRE and MTG. In addition, mitochondria in which  $\Delta\Psi$  was tracked (Fig. 3A, S3C-E) moved in the cell but maintained stable  $\Delta\Psi$ , this further indicating low spatial variability in imaging measurements. Furthermore, there was no difference in  $\Delta\Psi$  between perinuclear and peripheral mitochondria.

*Temporal variability in  $\Delta\Psi$  at the single mitochondrion:* The changes in  $\Delta\Psi$  measured from the individual mitochondrion over time are a sum of noise due to imaging artefacts, biological artefacts and real changes in  $\Delta\Psi$ . Thus, we rationalized that the overall noise could not exceed the variability measured over time from a single mitochondrion in the intact cell (Fig. 3A). Measurements obtained from individual mitochondria in INS1 cells over time demonstrated remarkable stability (Fig. S3C-E). Mitochondria spent 70 % of their time at  $\pm 1.3$  mV around their baseline and less than 3 % of their time at  $\pm 2.7$  mV off their baseline ( $n = 11$ ).

#### **Additional considerations of the imaging technique**

In this study, we present a method to quantify heterogeneity in mV by simultaneously labelling mitochondria in intact cells with a combination of  $\Delta\Psi$ -dependent and  $\Delta\Psi$ -independent dyes and use their ratio for determining the range of  $\Delta\Psi$  within a given cell. An important advantage of using the TMRE/MTG ratio is the compensation for focal plane

variability in confocal sectioning (Fig. 1D). Further, the TMRE/MTG ratio adjusts for intracellular mitochondrial movement during time-lapse imaging (Fig. 2B-E, 3A, S3C-E).

The accuracy of MTG as a potential independent probe for mitochondrial mass is controversial, and apparently depends on cell-type (6, 7). We therefore examined the independence of MTG from  $\Delta\Psi$  in our cell lines by applying glucose, which hyperpolarize  $\Delta\Psi$ , and the depolarizing agent FCCP and observed the response in MTG FI. While MTG FI response to these agents was indelible, we caution that future studies consider this potentially cell-type dependent effect (Fig. 1B-C).

As experimental variables including laser intensity, detector sensitivity, and dye penetration / extrusion may influence FI,  $\Delta\Psi$  quantification facilitates inter-study comparisons. The  $\Delta\Psi$  response we observed to OM, a mitochondrial hyperpolarizing agent, are consistent with quantitative data previously reported (8).

## Supplements References

1. **Deutsch M, Deutsch A, Shirihai O, Hurevich I, Afrimzon E, Shafran Y, Zurgil N.** A novel miniature cell retainer for correlative high-content analysis of individual untethered non-adherent cells. *Lab Chip*. 6(8): 995-1000, 2006.
2. **O'Reilly CM, Fogarty KE, Drummond RM, Tuft RA, Walsh JV Jr.** Quantitative analysis of spontaneous mitochondrial depolarizations. *Biophys.J.* 85: 3350-3357, 2003.
3. **Nicholls DG, Ward MW.** Mitochondrial membrane potential and neuronal glutamate excitotoxicity: mortality and millivolts. *Trends Neurosci.* 23: 166-174, 2000.
4. **Collins TJ, Berridge MJ, Lipp P, Bootman MD.** Mitochondria are morphologically and functionally heterogeneous within cells. *EMBO J.* 21: 1616-1627, 2002.
5. **Vander Heiden MG, Chandel NS, Williamson EK, Schumacker PT, Thompson CB.** Bcl-xL regulates the membrane potential and volume homeostasis of mitochondria. *Cell*. 28;91 (5):627-37, 1997.
6. **Pendergrass W, Wolf N, Poot M.** Efficacy of MitoTracker Green and CMXRosamine to measure changes in mitochondrial membrane potentials in living cells and tissues. *Cytometry* 61A: 162-169, 2004.
7. **Keij JF, Bell-Prince C, Steinkamp JA.** Staining of mitochondrial membranes with 10-nonyl acridine orange, MitoFluor Green, and MitoTracker Green is affected by mitochondrial membrane potential altering drugs. *Cytometry* 39: 203-210, 2000.
8. **Ward MW, Rego AC, Frenguelli BG, Nicholls DG.** Mitochondrial membrane potential and glutamate excitotoxicity in cultured cerebellar granule cells. *J.Neurosci.* 20 (19) 7208-7219, 2000.
9. **Gao W, Pu Y, Luo KQ, Chang DC.** Temporal relationship between cytochrome c release and mitochondrial swelling during UV-induced apoptosis in living HeLa cells. *J.Cell Sci.* 114(15):2855-62, 2001.
10. **Quesada I, Todorova MG, Soria B.** Different metabolic responses in alpha, beta and delta-cells of the islet of Langerhans monitored by redox confocal microscopy. *Biophys.J.:* 90 (7): 2641-50 2006.

## Supplements Figures legends

### Fig. S1

Determining the fraction of  $\beta$ -cells hyperpolarizing in response to glucose. Cells were plated on the Optical LiveCell array (see bright-field (BF) image), a honeycomb shaped cell retainer, and stained with 7 nM TMRE (pseudo colour). Images were first obtained at 3 mM and 8 mM glucose, with a pause of 10 min in between (note the changes in fluorescence intensity in zoom image). After that, cells were fixed, immunostained for insulin and subsequently imaged (green image). Correlation of hyperpolarization to insulin staining revealed that 93 % of the cells responding to glucose were  $\beta$ -cells.

### Fig. S2

(A) Islet cell dispersion does not cause mitochondrial swelling. Distribution of mitochondrial diameter in whole islets versus dispersed islets.

(B) Glucolipototoxicity does not cause mitochondrial swelling. Distribution of mitochondrial diameter in cells under glucolipototoxicity (GLT) versus normal cells (Ctrl).

(C) INS1 cells exhibit similar heterogeneity in  $\Delta\Psi$ . Reprehensive pseudo-color image.

(D) Swelling and dilution of TMRE is not a contributor to heterogeneity. Correlation of mitochondrial size and  $\Delta\Psi$  (as TMRE/MTG FI ratio). Mitochondrial area and FI was measured in confocal images of  $\beta$ -cell mitochondria stained with MTG and TMRE (n = 134 mitochondria in 12 cells). The mitochondrial population was divided to depolarized and hyperpolarized based on a cut-off (400 [au], range 61-832) at 1 SD from the average towards the depolarization. The size distribution of mitochondria that were more depolarized (black) or hyperpolarized (red) than the cut-off value is shown. Note that depolarized mitochondria tend to be smaller, probably due to fragmentation.

### Fig. S3

(A) Minimal change in normalized heterogeneity value between two consecutive scans (n = 10 cells).

(B) Fluorescent beads exhibit low variability in fluorescence. Normalized ratio intensity values are indicated for each bead. Reprehensive pseudo-color image.

(C-E) Tracking  $\Delta\Psi$  in individual INS1 cell mitochondria using TMRE and matrix-targeted photo-activatable GFP. (C) A mitochondrion stained with TMRE and labelled with GFP by photo-activation. (D)  $\Delta\Psi$  of a single mitochondrion monitored by time lapse imaging. (E) Probability of deviation from baseline  $\Delta\Psi$  in single mitochondria. 70 % of their time is spent at  $\pm 1.3$  mV and only 3 % at  $\pm 2.7$  mV of their baseline (n = 11).