

Perspectives on: SGP Symposium on Mitochondrial Physiology and Medicine

The renaissance of mitochondrial pH

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The generation of a proton gradient across the inner mitochondrial membrane (IMM) is an essential energy conservation event that couples the oxidation of carbohydrates and fat to the synthesis of ATP. Studies in isolated mitochondria have established that the chemical gradient for protons ($\Delta p\text{H}_m$) and the mitochondrial membrane potential ($\Delta\Psi_m$) contribute independently to the proton-motive force (Δp) that drives the synthesis of ATP. Because $\Delta\Psi_m$ contributes most of the Δp and can be easily measured in intact cells with fluorescent dyes, most studies ignore the contribution of $\Delta p\text{H}_m$ and only record changes in $\Delta\Psi_m$ to track the metabolic state of mitochondria. $\Delta p\text{H}_m$, however, drives the fluxes of metabolic substrates required for mitochondrial respiration and the activity of electroneutral ion exchangers that maintain mitochondria osmolarity and volume, and recent studies indicate that the mitochondrial pH (pH_{mito}) plays an important and underappreciated role in physiological and pathological situations such as apoptosis, neurotransmission, and insulin secretion. In this Perspective, we discuss the putative roles of the pH_{mito} and review the different techniques used to measure pH_{mito} and $\Delta p\text{H}_m$ in isolated mitochondria and in intact cells, focusing on our recent results obtained with genetically encoded pH-sensitive indicators. These measurements have revealed that the pH_{mito} is in dynamic equilibrium with the cytosolic pH and that spontaneous pH_{mito} elevations coinciding with $\Delta\Psi_m$ drops occur in single mitochondria. Unlike the “superoxide flashes” reported with a pH-sensitive circularly permuted YFP (cpYFP), these “pH flashes” preserve the Δp during spontaneous fluctuations in $\Delta\Psi_m$; therefore, we propose that the flashes are energy conservation events that reflect the intrinsic properties of the mitochondrial proton circuit.

Introduction

Mitochondria are multifunctional organelles involved in energy conversion, lipid metabolism, heat production, Ca^{2+} signaling, reactive oxygen species (ROS) production, and apoptosis. All of these functions rely on the ability of mitochondria to move protons across their inner membrane during oxidative phosphorylation (OXPHOS), the process that couples the oxidation of energetic substrates to the synthesis of ATP. According to the chemiosmotic theory first postulated by Mitchell (1975), the free energy (ΔG) released by the oxidation of highly reduced energetic substrates is used by the complexes I, III, and IV of the electron transport chain to generate a proton gradient across the IMM. The energy stored in the proton gradient is then used to drive the activity of the ATP synthase (complex V) that catalyzes the conversion of ADP to ATP within the mitochondrial matrix. The importance of mitochondrial proton transport is highlighted by the retention of genes coding for OXPHOS subunits within the mitochondrial genome. Mitochondria are endosymbiotic organelles, and virtually all of the $\sim 1,500$ genes required to build a functional mitochondria have been transferred to the chromosomes of the host cell, except for those coding for 13 polypeptides of the OXPHOS subunits, plus the ribosomal and transfer RNAs required for their synthesis. The 13 mitochondrial-encoded proteins include seven subunits of the respiratory chain complex I, one of complex III, three of complex IV, and two of the complex V, i.e., all the respiratory chain complexes that are involved in the transport of protons. The chemiosmotic theory is rooted in measurements of bioenergetics parameters, such as oxygen consumption, ATP production, pH, and membrane potential, in isolated mitochondria artificially maintained under different metabolic conditions. In intact cells, however, mitochondria are exposed to metabolic and environmental fluctuations, interact with other organelles, and receive inputs from cell signaling pathways. Therefore, data derived from experiments in isolated mitochondria cannot be readily transposed *in vivo*.

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Abbreviations used in this paper: CHX, Ca^{2+} - H^+ exchanger; cpYFP, circularly permuted YFP; Δp , proton-motive force; $\Delta p\text{H}_m$, chemical gradient for protons; $\Delta\Psi_m$, mitochondrial membrane potential; IMM, inner mitochondrial membrane; IMS, intermembrane space; KHX, K^+ - H^+ exchanger; mPTP, permeability transition pore; NHX, Na^+ - H^+ exchanger; OXPHOS, oxidative phosphorylation; pH_{IMS} , pH within the IMS; pH_{mito} , mitochondrial pH; PiC, P_i - H^+ phosphate cotransporter; ROS, reactive oxygen species; UCP, uncoupling protein.

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In this Perspective, we will briefly describe the mechanism that maintains and regulates pH_{mito} as established in isolated mitochondria and integrate this knowledge with more recent recordings of pH_{mito} in intact living cells obtained with genetically encoded pH-sensitive probes, with a focus on our recent report that single mitochondria exhibit spontaneous pH_{mito} elevations.

Moving protons in and out of mitochondria

Given the central role of mitochondrial proton transport in energy conversion, much effort has been devoted to unraveling the sophisticated molecular machinery that moves protons across the IMM. Protons are extruded from the matrix to the intermembrane space (IMS) by the respiratory complexes I, III, and IV during consecutive redox reactions that couple the free energy released during the transport of electrons from high to low redox potentials to the extrusion of protons (Dempsey et al., 2010). Crystal structures of the respiratory complexes have been obtained (Abrahams et al., 1994; Iwata et al., 1998), and the stoichiometry of H^+ ejection was established as 10 H^+ pumped for each pair of electrons entering at the level of complex I (Saraste, 1999). Because of the low permeability of the IMM to ions, including H^+ , the extrusion of protons by the respiratory complexes creates an electrochemical gradient for H^+ across the membrane ($\Delta\tilde{\mu}_{\text{H}^+}$, more commonly expressed as Δp). Δp is the sum of an electrical gradient that constitutes the $\Delta\Psi_m$ and of a chemical gradient ΔpH_m that reflects the pH difference between the pH_{mito}

and the pH within the IMS (pH_{IMS}). From a chemiosmotic point of view, $\Delta\Psi_m$ and ΔpH_m are independent components that equally contribute to the Δp driving the synthesis of ATP as H^+ ions return to the matrix at the level of complex V (Mitchell, 1961). In addition to powering ATP synthesis, the potential energy stored in the H^+ gradient also drives the transport of ions and metabolites across the IMM (Fig. 1). Some transporters rely only on $\Delta\Psi_m$, for instance: Ca^{2+} uptake via the mitochondrial Ca^{2+} uniporter (Baughman et al., 2011; De Stefani et al., 2011), ATP-ADP exchange via the adenine nucleotide translocator (Krämer and Klingenberg, 1980; Klingenberg, 2008), or the import of mitochondrial resident protein via the translocase of outer membrane and the translocase of inner membrane complexes (Martin et al., 1991; Bauer et al., 1996). Conversely, several transporters rely exclusively on ΔpH_m , such as the Ca^{2+} - H^+ exchanger (CHX), K^+ - H^+ exchanger (KHX), and Na^+ - H^+ exchanger (NHX), whose molecular identities remain controversial (Nowikovsky et al., 2004; Jiang et al., 2009; Zotova et al., 2010). The P_i - H^+ phosphate cotransporter (PiC), which imports the phosphate required for ATP synthesis into the matrix, also relies on ΔpH_m (Palmieri, 2004). Some transporters dissipate both ΔpH_m and $\Delta\Psi_m$, such as uncoupling proteins (UCPs), H^+ channels that uncouple OXPHOS from ATP synthesis, and the permeability transition pore (mPTP), a nonselective ion channel whose opening initiates cell death by allowing the fluxes of ions and metabolites of up to 1,500 KD across the IMM

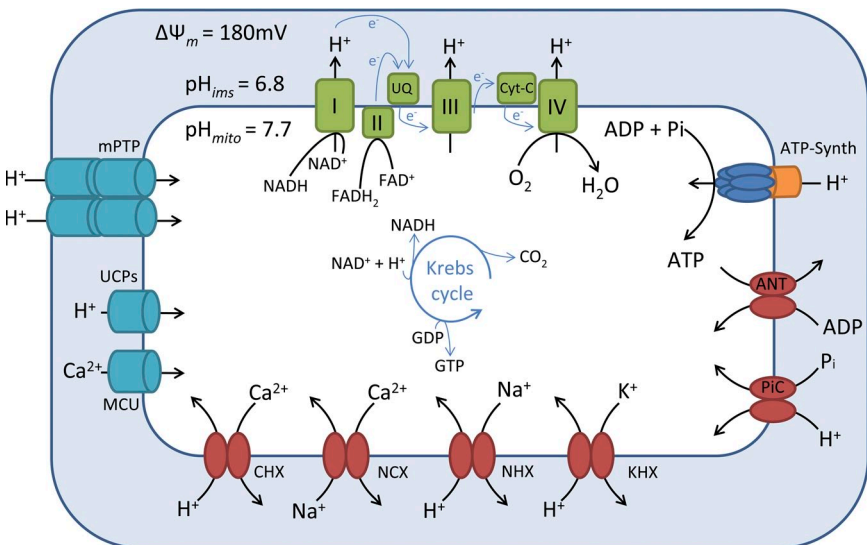


Figure 1. Determinants of the pH_{mito} . Protons are pumped from the matrix to the IMS by the respiratory chain complexes I, III, and IV (green boxes) as electrons flow from reduced substrates in the matrix to O_2 . The pumping of electrically charged protons generates a $\Delta\Psi_m$ of ~ 180 mV and a pH gradient (ΔpH_m : $\text{pH}_{\text{mito}} - \text{pH}_{\text{IMS}}$) of ~ 0.9 pH units as the matrix becomes more alkaline than the IMS. The proton circuit is in thermodynamic equilibrium and changes in $\Delta\Psi_m$, thus causing opposing changes in ΔpH_m by altering the energy required for the pumping of protons by respiratory chain complexes. $\Delta\Psi_m$ and ΔpH_m add up to generate a Δp used by the ATP synthase (blue-orange barrel) to generate ATP from ADP and P_i in the matrix. $\Delta\Psi_m$ drives Ca^{2+} uptake across the mitochondrial Ca^{2+} uniporter (MCU; blue cylinder) and ADP-ATP exchange across the adenine nucleotide translocator (ANT; brown ovals). Electroneutral H^+ -ion exchangers rely exclusively on ΔpH_m to extrude Ca^{2+} , Na^+ , and K^+ in exchange for protons (CHX, NHX, and KHX, respectively; brown ovals), whereas the PiC relies on ΔpH_m to import the inorganic phosphate used for the synthesis of ATP (PiC; brown ovals). The coupling of H^+ and ion fluxes implies that changes in the Na^+ , K^+ , Ca^{2+} , and P_i gradients can alter ΔpH_m . UCPs and the mPTP (UCPs and mPTP; blue cylinders) dissipate both ΔpH_m and $\Delta\Psi_m$ to generate heat and to initiate cell death, respectively. Variations in pH_{mito} reflect the equilibrium between proton pumping by the respiratory chain; Δp dissipation by the ATP synthase, UCPs, and mPTP; ΔpH_m dissipation by KHX, NHX, CHX, and PiC; and adaptive responses to changes in cytosolic pH and in $\Delta\Psi_m$.

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(Kroemer et al., 2007). UCP1 is expressed in brown adipose fat where it acts as a proton channel to mediate adaptive thermogenesis (Cannon and Nedergaard, 2004), whereas the UCP2 and UCP3 isoforms, expressed in non-adipose tissues, do not appear to function as proton channels under basal conditions (Cadenas et al., 2002; Couplan et al., 2002) but only upon stimulation by fatty acids and purine nucleotides (Palmieri, 2004). The mitochondrial matrix pH, pH_{mito} , reflects the equilibrium between proton extrusion and proton entry into the matrix. Variations in pH_{mito} therefore reflect the equilibrium between proton pumping by the respiratory chain and proton back-flux across the ATP synthase, across the KHX, NHX, CHX, and PiC, and across the UCP and mPTP. Variations in pH_{mito} are also limited by mitochondrial H^+ buffers provided by the side chains of amino acids and by phosphates and bicarbonates, which dampen the variations in the free H^+ concentration during acid or alkaline loads. But because the pH_{mito} -buffering capacity (β_{mito}) is quite low at the physiological alkaline pH of the matrix (Poburko et al., 2011), pH_{mito} changes mainly reflect the activity of H^+ fluxes across the IMM.

Measurements in isolated mitochondria

The validation of the chemiosmotic theory implied precise measurements of the electrical and chemical component of the Δp under well-controlled conditions, and until 1980, the preparation of choice was isolated mitochondria purified from liver by differential centrifugation. After attempts to impale giant mitochondria with microelectrodes (Maloff et al., 1977), physiologists relied on external K^+ and H^+ -selective electrodes or on isotopes to measure $\Delta\Psi_m$ and ΔpH_m in suspended mitochondria (Mitchell and Moyle, 1969). The electrical component $\Delta\Psi_m$ was estimated by measuring the distribution of radioactively labeled lipophilic cations or by recording the changes in external $[K^+]$ or the accumulation of matrix $^{86}Rb^+$ in the presence of the potassium ionophore valinomycin. This approach relies on the assumption that cations distribute according to the Donnan equilibrium and provided precise estimates of the distribution of K^+ or Rb^+ across the IMM. The chemical component ΔpH_m was estimated by measuring the distribution of radioactively labeled weak acids or bases, 3H -acetate or ^{14}C -methylamine, assuming that the IMM is permeable to the uncharged but impermeable to the charged species (Nicholls, 1974), or by monitoring the changes in external pH after the lysis of mitochondria with detergents to estimate pH_{mito} , a calculation that requires the knowledge of the mitochondrial volume and of the buffering capacity of the mitochondrial matrix (Rottenberg, 1975). These measurements established that Δp ranges from 180 to 220 mV depending on the metabolic state of the mitochondria, with $\Delta\Psi_m$ ranging from 150 to 180 mV and ΔpH_m from 0.5 to 1.2 pH units

($pH_{mito} = 8.2-7.5$ and $pH_{out} = 7$). Using the simplified Nernst equation ($E_H = -60 \log [H^+]_i/[H^+]_o$ at $30^\circ C$), the pH gradient can be converted into a diffusion potential and its contribution to the Δp was estimated to be $\sim 30-70$ mV, i.e., 17–30% of Δp , indicating that $\Delta\Psi_m$ is the main component of the Δp . These measurements provided the first quantitative estimates of the two components to the Δp generated by mitochondria, grounding the chemiosmotic theory in solid scientific evidence and confirming several of its predictions. One of these predictions was the postulate that $\Delta\Psi_m$ and ΔpH_m add up to build Δp , which implied that, in respiring mitochondria, selective manipulations of $\Delta\Psi_m$ would induce compensatory alterations in ΔpH_m to preserve Δp . This was nicely demonstrated by Nicholls (1974) in isolated mitochondria equilibrated with valinomycin/ K^+ and exposed to increasing amounts of K^+ to clamp $\Delta\Psi_m$ to varying voltages. In these conditions, Δp remains constant as mitochondria are depolarized because the decreases in $\Delta\Psi_m$ are exactly balanced by opposite increases in ΔpH_m (see Nicholls, 2005, for a recent discussion of these findings). The compensation occurs over the whole range of voltages tested to the point that, when $\Delta\Psi_m$ is fully dissipated, the Δp is contributed exclusively by ΔpH_m . Conversely, when ΔpH_m is collapsed by the K^+/H^+ ionophore nigericin, Δp is contributed exclusively by $\Delta\Psi_m$ (Lambert and Brand, 2004). These experiments demonstrated that the two components of the Δp can vary widely without dissipating the stored energy, as ΔpH_m can fully compensate for imposed changes in $\Delta\Psi_m$ and vice versa.

Measurements in living cells

The recognition that $\Delta\Psi_m$ is the major contributor of Δp fostered the development of optical recording techniques to measure this parameter in intact cells. Since 1980, the preferred method is to use fluorescence lipophilic cations that distribute across the IMM according to the membrane potential. Fluorescent dyes such as TMRM, rhodamine, or JC1 provide a simple optical readout of the mitochondrial potential and enable the study of its dynamic regulation in intact living cells under physiological conditions. These dyes have become so popular that, in virtually all studies, the fluorescent $\Delta\Psi_m$ signal is thought to reflect the energization state of mitochondria, an assumption that equates $\Delta\Psi_m$ with Δp and thus ignores the contribution of ΔpH_m . As discussed in the preceding section, however, ΔpH_m contributes 20–30% of Δp and can fully compensate for a loss in $\Delta\Psi_m$ when the mitochondrial potential is varied with an artificial K^+ conductance. To confidently establish the energization state of mitochondria, both $\Delta\Psi_m$ and ΔpH_m should be measured simultaneously to obtain a complete readout of Δp . Unfortunately, ΔpH_m is not only ignored but also more difficult to measure than $\Delta\Psi_m$, and very few studies so far have attempted to record

dynamic changes in ΔpH_m . Using radioactively labeled weak acid and bases, ΔpH_m was estimated around 1.0–1.2 pH units, contributing 60 mV to Δp in intact cells (Hoek et al., 1980; Brand and Felber, 1984), but isotopic measurements are restricted to cell populations, do not allow real-time recordings, and do not provide any spatial information. Optical recordings of pH_{mito} with pH-sensitive fluorescent dyes such as BCECF or SNARF brought the resolution down to the single-cell level. Using this approach, ΔpH_m was found to be ~ 0.9 pH units in cardiac myocytes and to collapse with a different kinetic than $\Delta\Psi_m$ during chemical hypoxia (Lemasters et al., 1995), whereas in MDCK cells, ΔpH_m was around 0.3 pH units and was dynamically regulated during metabolic inhibition (Balut et al., 2008). Because chemical dyes are not specifically targeted to mitochondria, cells must be simultaneously loaded with a fluorescent mitochondrial marker to distinguish between the mitochondrial and cytosolic pH signal; therefore, this approach is better suited for isolated mitochondria or permeabilized cells. To enable time-resolved *in situ* recordings of pH_{mito} , an ideal fluorescent sensor should exhibit the following properties: (a) specific targeting to the mitochondrial matrix; (b) reduced toxicity compared with BCECF-AM or SNARF-AM, which generate harmful metabolites and produce ROS when excited by light; (c) rapid and reversible response to variations in pH_{mito} ; (d) alkaline pK_a around 7.6–8.0 to match the pH of the mitochondrial matrix; (e) wide dynamic range to reveal small changes in pH_{mito} levels between individual mitochondria; (f) high pH specificity to discriminate between pH changes and changes in ionic strength or in redox conditions; (g) ratiometric to avoid confounding factors caused by imaging conditions, cell thickness, or probe expression levels; and (h) available in different spectral variants to facilitate simultaneous pH measurements in different compartments or the monitoring of other mitochondrial parameters with probes of distinct spectral properties.

The development of genetically encoded pH-sensitive indicators solves the targeting issue because the protein-based probes can be specifically targeted to specific organelles with endogenous addressing sequences. These probes are derived from the GFP, a molecule whose fluorescence properties are well understood at the molecular level. The GFP chromophore originates by spontaneous posttranslational cyclization of three consecutive amino acids located inside the hydrophobic environment created by 11-stranded β sheets that form the characteristic β -barrel tertiary structure of the protein. Because their spectral properties depend on the protonation state of the chromophore, GFPs can be easily turned into pH sensors by mutating residues that alter the conformation of the chromophore or its accessibility to solvent (Miesenböck et al., 1998; Hanson et al., 2002). The initial pH-sensitive GFP mutants have

a pK_a in the acidic or near-neutral range and are therefore best suited for measurements in acidic organelles (Kneen et al., 1998; Miesenböck et al., 1998), but pH_{mito} acidification evoked by protonophores could be detected with mitochondrial versions of the GFP mutant F64L/S65T (Kneen et al., 1998) and with mito-EYFP (Llopis et al., 1998). Using a pH-sensitive GFP, a mitochondrial alkalization concomitant with a cytosolic acidification was reported in apoptotic cells and attributed to the reverse activity of the ATP synthase (i.e., pumping H^+ toward the cytosol), the ensuing cytosolic acidification favoring the activity of caspases and promoting apoptosis (Matsuyama et al., 2000). Different ratiometric and nonratiometric GFP mutants have since been developed that exhibit an alkaline-shifted pK_a , such as the YFP mutants H148G ($\text{pK}_a = 8$) and S65T/H114D ($\text{pK}_a = 7.8$) (Elslinger et al., 1999), or the deGFP1 S65T/H114G/T203C ($\text{pK}_a = 8$) (Hanson et al., 2002). Using another strategy, Pozzan's group (Abad et al., 2004) took advantage of the high pH sensitivity of the YFP-based Ca^{2+} sensors Camgaroos to generate a probe with an apparent pK_a of 8.5, mt-AlpHi, by replacing the Ca^{2+} -sensitive domain of the Camgaroo by a Ca^{2+} -insensitive module. In HeLa cells and primary cultured neurons, mt-AlpHi reported that basal pH_{mito} levels were around 8.0 and increased heterogeneously upon stimulation with Ca^{2+} -mobilizing agonists, with some mitochondria alkalinizing and others not (Abad et al., 2004). In rat pancreatic β cells, sustained increases in pH_{mito} and in ΔpH_m were observed with mt-AlpHi during glucose stimulation that correlated with an increase in mitochondrial ATP synthesis, indicating that pH_{mito} is an important signal during nutrient-induced insulin secretion (Wiederkehr et al., 2009). Treatment with nigericin to prevent pH_{mito} alkalization blunted nutrient-induced ATP increase and insulin secretion (Akhmedov et al., 2010), indicating that pH_{mito} and ΔpH_m control mitochondrial metabolism during cell stimulation (Wiederkehr, 2009). The new red-shifted RFPs are also promising tools to measure pH in living cells (Johnson et al., 2009), although their pK_a values in the acidic range preclude accurate pH measurements in alkaline organelles (Jach et al., 2006; Shaner et al., 2008). pHRed ($\text{pK}_a = 7.8$) has been used to carry out simultaneous measurements of pH_{cyto} and ATP_{cyto} combined with Perceval (Tantama et al., 2011), and the availability of such alkaline-sensitive red-shifted fluorescent proteins will allow multicolor imaging of pH_{mito} together with key parameters like Ca^{2+} , ATP, or ROS.

ΔpH_m is usually calculated as $\text{pH}_{\text{mito}} - \text{pH}_{\text{cyto}}$ because the outer mitochondrial membrane has a high permeability to ions. The bulk pH_{cyto} , however, might not reflect the actual pH values achieved in the IMS, where H^+ is continuously ejected by respiratory chain complexes. Accordingly, recordings with a pH-sensitive YFP targeted to the outer surface of the IMM reported a pH_{IMS} of 6.8, i.e.,

slightly more acidic than the cytosol, and a ΔpH_m of 0.8 pH units (Porcelli et al., 2005). The pH on the IMS side of mitochondria cristae might be even more acidic than the bulk IMS pH because respiratory complexes are concentrated on these invaginations, which are connected to the IMS by small tubular junctions that constrain the diffusion of solutes (Scorrano et al., 2002). Indeed, electron cryotomography studies reported long ribbons of ATP synthase dimers assembling on tightly curved cristae edges (Strauss et al., 2008), an arrangement predicted to increase the surface density of protons in the curved membrane regions by ~ 0.5 pH units, thereby turning cristae into proton traps (Davies et al., 2011).

We have recently developed a new genetically encoded pH-sensitive probe, mito-SypHer, which we used to follow ΔpH_m changes during physiological activation of cells by Ca^{2+} -mobilizing agonists (Poburko et al., 2011). The probe was derived from HyPer, a cpYFP-based indicator for hydrogen peroxide very sensitive to alkaline pH, by mutating a cysteine residue to remove the probe H_2O_2 sensitivity. SypHer is highly sensitive to pH but insensitive to oxidizing and reducing agents, and has two maximal absorbance peaks at 430 and 490 nm that enable ratiometric measurements of the changes in environmental pH. By combining mito-SypHer with a fluorescent pH dye, we could record pH_{mito} and pH_{cyto} simultaneously to track dynamic changes in ΔpH_m in live cells. In HeLa cells, pH_{mito} and ΔpH_m averaged 7.6 and 0.45 and, surprisingly, decreased together with pH_{cyto} during activation of cells with Ca^{2+} -mobilizing agonists (Fig. 2). The rapid acidification of the cytosol reflected the activity of plasma membrane Ca^{2+} pumps, and the cytosolic acid was readily transmitted to the mitochondrial matrix, predominantly via the KHX and Pi/H^+ symporter, thereby causing a mitochondrial acidification instead of the alkalization that was previously reported with mt-AlpHi in HeLa cells exposed to histamine (Abad et al., 2004) and in pancreatic β cells treated with glucose (Wiederkehr et al., 2009). The ΔpH_m decrease reflected the larger decrease in pH_{mito} compared with pH_{cyto} (Fig. 2), which in turn reflects the lower buffering capacity of mitochondria at physiological pH levels ($\beta_{\text{mito}} = 5$ mM at pH 7.8) compared with the cytosol ($\beta_{\text{cyto}} = 20$ mM at pH 7.4). Similar matrix acidification and ΔpH_m dissipation were observed in astrocytes exposed to glutamate, with the decreased ΔpH_m being associated with decreased O_2 consumption and reduced mitochondrial ROS generation (Azarias et al., 2011), suggesting that the mitochondrial metabolism of astrocytes decreases during neurotransmission, a mechanism that might increase local oxygen availability for neurons. The matrix acidification and ΔpH_m dissipation observed in HeLa cells and astrocytes appears at odds with earlier studies showing that cytosolic Ca^{2+} elevations boost mitochondrial metabolism (Hajnóczky et al., 1995), but the rapid acidification evoked by the cytosolic Ca^{2+}

elevations was followed by a slow matrix alkalization as the cytosolic Ca^{2+} signal subsided (Fig. 2), consistent with Ca^{2+} -dependent activation of matrix enzymes. Furthermore, the addition of micromolar Ca^{2+} concentrations to permeabilized cells induced a slight and progressive matrix alkalization (Poburko et al., 2011). These findings suggest that cytosolic Ca^{2+} elevations exert opposite effects on pH_{mito} , as they stimulate mitochondrial respiration, thereby increasing pH_{mito} , and at the same time generate large quantities of cytosolic acid that is transmitted to the mitochondrial matrix, thereby decreasing pH_{mito} . In cells that are essentially glycolytic such as cultured HeLa cells and astrocytes, the latter mechanism dominates and ΔpH_m decreases during Ca^{2+} elevations. More fundamentally, these data indicate that the permeability of the IMM to protons is quite high in situ and thus appear to contradict the fourth postulate of the chemiosmotic theory, that mitochondria must be impermeable to protons to allow the generation of a Δp . However, the rapid pH equilibration was not caused by electrophoretic entry of protons but by the activity of electroneutral ion- H^+ exchangers, and our findings therefore remain consistent with the chemiosmotic theory, whose third postulate predicts the existence of exchangers coupling anion entry and cation extrusion to proton entry.

pH elevations in single mitochondria

Advances in live cell imaging revealed that mitochondria are morphologically and functionally heterogeneous within cells (Collins et al., 2002) and that rapid

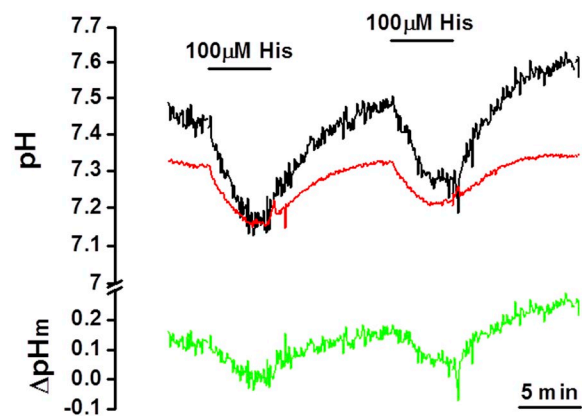


Figure 2. Dynamic recordings of ΔpH_m during cell activation. Simultaneous recordings of pH_{mito} (black trace, mito-SypHer) and pH_{cyto} (red trace, SNARF) in HeLa cells repeatedly stimulated with 100 μM histamine to elicit Ca^{2+} elevations. ΔpH_m can be calculated online as $\text{pH}_{\text{mito}} - \text{pH}_{\text{cyto}}$ (green trace). Both pH_{cyto} and pH_{mito} decrease during Ca^{2+} elevations as large quantities of cytosolic acid are generated by the activity of plasma membrane Ca^{2+} pumps. The larger decrease in pH_{mito} reflects the lower H^+ -buffering capacity of mitochondria and causes ΔpH_m to collapse at the peak of the Ca^{2+} elevations. pH_{mito} and ΔpH_m increased upon histamine removal, reflecting the Ca^{2+} -dependent activation of matrix dehydrogenases. See also Poburko et al. (2011).

fluctuations in $\Delta\Psi_m$ occur in single mitochondria (Duchen et al., 1998; Hüser et al., 1998). The depolarization transients have been proposed to be triggered by Ca^{2+} elevations (Duchen et al., 1998), by openings of the mPTP (Hüser and Blatter, 1999; De Giorgi et al., 2000; Zorov et al., 2000; Jacobson and Duchen, 2002), by changes in the matrix concentration of adenine nucleotides (Vergun et al., 2003; Vergun and Reynolds, 2004), by the activity of the ATP synthase (Thiffault and Bennett, 2005), or by the opening of a H^+ -selective channel (Hattori et al., 2005), and their functional significance is currently unknown. The fluctuations in $\Delta\Psi_m$ coincide with transient elevations in matrix $[\text{Na}^+]$ in astrocytes (Azarias et al., 2008), with ROS oscillations and NADH fluctuations in cardiac myocytes (Aon et al., 2003), and with superoxide flashes in skeletal muscle and intact beating hearts (Wang et al., 2008; Pouvreau, 2010; De Stefani et al., 2011). The nature of the superoxide flashes is debated because flash activity persisted under anaerobic conditions and was abolished by all respiratory chain inhibitors including antimycin, which is known to boost superoxide production (Muller, 2009). In response to these criticisms, the authors performed additional experiments to show that the flashes are nearly abrogated during chemical and physical anoxia, and attributed the unexpected effects of antimycin to the unique mechanism of superoxide flash production (Huang et al., 2011). In plants, the cpYFP probe used to detect the putative superoxide flashes was found to be highly responsive to changes in matrix pH but insensitive to changes in matrix superoxide, raising the possibility that the fluctuations were pH and not superoxide

flashes (Schwarzländer et al., 2011). Using our ratio-metric pH-sensitive probe mito-SypHer, we and others observed spontaneous pH_{mito} elevations of 0.4 pH units coinciding with decreases in $\Delta\Psi_m$ in individual mitochondria of HeLa cells (Fig. 3) (Santo-Domingo, J., and N. Demaurex. 2010. 16th European Bioenergetics Conference. Abstr. 15L.3; Santo-Domingo, J., and N. Demaurex. 2011. 65th Annual Meeting of The Society of General Physiologists. Abstr. 34) and of astrocytes (Azarias and Chatton, 2011). The pH_{mito} elevations had an abrupt onset and a slower recovery and their frequency was reduced by all respiratory chain inhibitors, a spatiotemporal and pharmacological profile similar to the superoxide flashes. To clarify the nature of the signal, we tested the pH and superoxide sensitivity of bacterially expressed SypHer and found the probe to be highly sensitive to pH but insensitive to superoxide in vitro (Santo-Domingo, J., and N. Demaurex. 2012. Biophysical Society 56th Annual Meeting. Abstr. 2907). Increasing the pH-buffering power of mitochondria delayed and decreased the amplitude of the pH_{mito} elevations, strongly suggesting that the elevations were caused by protons. Although this manipulation could alter mitochondrial function, it is unlikely to distort the kinetics of superoxide flashes exactly as predicted from the increase in pH-buffering power (Poburko et al., 2011). The rapid and transient elevations in SypHer ratio fluorescence observed in single mitochondria therefore reflect increases in matrix pH. Interestingly, we observed that enforced mitochondrial fusion increased the spatial extent of the pH_{mito} elevations, whereas fragmentation had the opposite effect, indicating that

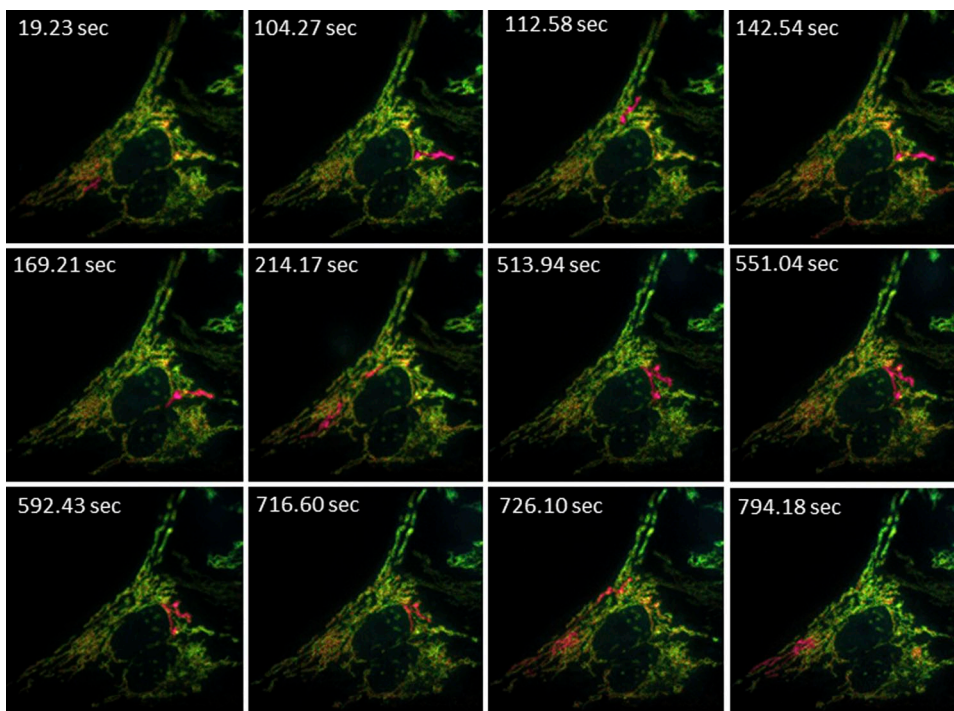


Figure 3. Alkalinization transients in single mitochondria. HeLa cells expressing mito-SypHer were recorded on a spinning disc confocal microscope at a frequency of 1.2 Hz. Ratio F480/F430 images from two cells exhibiting spontaneous alkalinization transients are shown, with warm colors denoting high ratio values. The pH_{mito} elevations occurred either in different regions of the mitochondrial network or repeatedly at the same location, but they always remained restricted to a particular mitochondrial cluster.

mitochondrial fusion facilitates the propagation of ΔpH_m by functionally coupling mitochondria. The pH_{mito} elevations persisted in cells permeabilized with solutions devoid of ions and, importantly, could be mimicked by artificial depolarization of mitochondria. These observations indicate that the pH_{mito} flashes, which occur coincidentally with spontaneous decreases in $\Delta\Psi_m$, reflect increased pumping by the respiratory chain during drops in $\Delta\Psi_m$. A transient mitochondrial depolarization thermodynamically favors H^+ extrusion by decreasing the driving force for proton pumping by the respiratory chain complexes, and several studies in isolated mitochondria have confirmed this prediction by showing that an imposed decrease in $\Delta\Psi_m$ increases the rate of proton extrusion and O_2 consumption (Talbot et al., 2007). Therefore, pH_{mito} flashes reflect the intrinsic properties of the mitochondrial proton circuit. These findings have important functional consequences, because other studies have linked superoxide flashes to altered mitochondrial respiration during oxidative stress-induced apoptosis (Ma et al., 2011). We propose instead that the flashes are alkalinization events that do not alter the ability of mitochondria to convert energy but that, on the contrary, preserve the Δp during spontaneous fluctuations in $\Delta\Psi_m$. Spontaneous $\Delta\Psi_m$ fluctuations are a well-known phenomenon thought to reflect alterations in mitochondrial metabolism. The observation that the Δp remains constant during concomitant $\Delta\Psi_m$ drops and pH_{mito} flashes indicates that the ability of mitochondria to convert energy is preserved during these bursts of electrical and chemical activity.

In conclusion, the pH_{mito} , which was long neglected, is the object of renewed interest as GFP-based pH-sensitive indicators now allow recordings of dynamic changes in pH_{mito} in living cells. The interpretation of pH_{mito} changes is difficult because the steady-state pH of the organelle reflects the combined activities of the respiratory chain and of mitochondrial H^+ transporters and is affected by variations in cytosolic pH and by variations in $\Delta\Psi_m$. The observation that elementary fluctuations in ΔpH_m occur in single mitochondria and spread across the cell as mitochondria fuse provides new insights on the properties of the mitochondrial proton circuit and on the ability of mitochondria to propagate energy inside cells.

This Perspectives series includes articles by Sheu et al., Zhang et al., Balaban, Wei and Dirksen, O-Uchi et al., Nowikovsky et al., and Galloway and Yoon.

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REFERENCES

- Abad, M.F., G. Di Benedetto, P.J. Magalhães, L. Filippin, and T. Pozzan. 2004. Mitochondrial pH monitored by a new engineered green fluorescent protein mutant. *J. Biol. Chem.* 279:11521–11529. <http://dx.doi.org/10.1074/jbc.M306766200>
- Abrahams, J.P., A.G. Leslie, R. Lutter, and J.E. Walker. 1994. Structure at 2.8 Å resolution of F1-ATPase from bovine heart mitochondria. *Nature*. 370:621–628. <http://dx.doi.org/10.1038/370621a0>
- Akhmedov, D., M. Braun, C. Matak, K.S. Park, T. Pozzan, K. Schoonjans, P. Rorsman, C.B. Wollheim, and A. Wiederkehr. 2010. Mitochondrial matrix pH controls oxidative phosphorylation and metabolism-secretion coupling in INS-1E clonal beta cells. *FASEB J.* 24:4613–4626. <http://dx.doi.org/10.1096/fj.10-162222>
- Aon, M.A., S. Cortassa, E. Marbán, and B. O’Rourke. 2003. Synchronized whole cell oscillations in mitochondrial metabolism triggered by a local release of reactive oxygen species in cardiac myocytes. *J. Biol. Chem.* 278:44735–44744. <http://dx.doi.org/10.1074/jbc.M302673200>
- Azarias, G., and J.Y. Chatton. 2011. Selective ion changes during spontaneous mitochondrial transients in intact astrocytes. *PLoS ONE*. 6:e28505. <http://dx.doi.org/10.1371/journal.pone.0028505>
- Azarias, G., D. Van de Ville, M. Unser, and J.Y. Chatton. 2008. Spontaneous Na^+ transients in individual mitochondria of intact astrocytes. *Glia*. 56:342–353. <http://dx.doi.org/10.1002/glia.20619>
- Azarias, G., H. Perreten, S. Lengacher, D. Poburko, N. Demaurex, P.J. Magistretti, and J.Y. Chatton. 2011. Glutamate transport decreases mitochondrial pH and modulates oxidative metabolism in astrocytes. *J. Neurosci.* 31:3550–3559. <http://dx.doi.org/10.1523/JNEUROSCI.4378-10.2011>
- Balut, C., M. vandeVen, S. Despa, I. Lambrichts, M. Ameloot, P. Steels, and I. Smets. 2008. Measurement of cytosolic and mitochondrial pH in living cells during reversible metabolic inhibition. *Kidney Int.* 73:226–232. <http://dx.doi.org/10.1038/sj.ki.5002632>
- Bauer, M.F., C. Sirrenberg, W. Neupert, and M. Brunner. 1996. Role of Tim23 as voltage sensor and presequence receptor in protein import into mitochondria. *Cell*. 87:33–41. [http://dx.doi.org/10.1016/S0092-8674\(00\)81320-3](http://dx.doi.org/10.1016/S0092-8674(00)81320-3)
- Baughman, J.M., F. Perocchi, H.S. Girgis, M. Plovanich, C.A. Belcher-Timme, Y. Sancak, X.R. Bao, L. Strittmatter, O. Goldberger, R.L. Bogorad, et al. 2011. Integrative genomics identifies MCU as an essential component of the mitochondrial calcium uniporter. *Nature*. 476:341–345. <http://dx.doi.org/10.1038/nature10234>
- Brand, M.D., and S.M. Felber. 1984. Membrane potential of mitochondria in intact lymphocytes during early mitogenic stimulation. *Biochem. J.* 217:453–459.
- Cadenas, S., K.S. Echtay, J.A. Harper, M.B. Jekabsons, J.A. Buckingham, E. Grau, A. Abuin, H. Chapman, J.C. Clapham, and M.D. Brand. 2002. The basal proton conductance of skeletal muscle mitochondria from transgenic mice overexpressing or lacking uncoupling protein-3. *J. Biol. Chem.* 277:2773–2778. <http://dx.doi.org/10.1074/jbc.M109736200>
- Cannon, B., and J. Nedergaard. 2004. Brown adipose tissue: function and physiological significance. *Physiol. Rev.* 84:277–359. <http://dx.doi.org/10.1152/physrev.00015.2003>
- Collins, T.J., M.J. Berridge, P. Lipp, and M.D. Bootman. 2002. Mitochondria are morphologically and functionally heterogeneous within cells. *EMBO J.* 21:1616–1627. <http://dx.doi.org/10.1093/emboj/21.7.1616>
- Couplan, E., M. del Mar Gonzalez-Barroso, M.C. Alves-Guerra, D. Ricquier, M. Gubern, and F. Bouillaud. 2002. No evidence for a basal, retinoic, or superoxide-induced uncoupling activity of the uncoupling protein 2 present in spleen or lung mitochondria. *J. Biol. Chem.* 277:26268–26275. <http://dx.doi.org/10.1074/jbc.M202535200>

- Davies, K.M., M. Strauss, B. Daum, J.H. Kief, H.D. Osiewacz, A. Rycovska, V. Zickermann, and W. Kühlbrandt. 2011. Macromolecular organization of ATP synthase and complex I in whole mitochondria. *Proc. Natl. Acad. Sci. USA*. 108:14121–14126. <http://dx.doi.org/10.1073/pnas.1103621108>
- De Giorgi, F., L. Lartigue, and F. Ichas. 2000. Electrical coupling and plasticity of the mitochondrial network. *Cell Calcium*. 28:365–370. <http://dx.doi.org/10.1054/ceca.2000.0177>
- De Stefani, D., A. Raffaello, E. Teardo, I. Szabò, and R. Rizzuto. 2011. A forty-kilodalton protein of the inner membrane is the mitochondrial calcium uniporter. *Nature*. 476:336–340. <http://dx.doi.org/10.1038/nature10230>
- Dempsey, J.L., J.R. Winkler, and H.B. Gray. 2010. Proton-coupled electron flow in protein redox machines. *Chem. Rev.* 110:7024–7039. <http://dx.doi.org/10.1021/cr100182b>
- Duchen, M.R., A. Leyssens, and M. Crompton. 1998. Transient mitochondrial depolarizations reflect focal sarcoplasmic reticular calcium release in single rat cardiomyocytes. *J. Cell Biol.* 142:975–988. <http://dx.doi.org/10.1083/jcb.142.4.975>
- Elsiger, M.A., R.M. Wachter, G.T. Hanson, K. Kallio, and S.J. Remington. 1999. Structural and spectral response of green fluorescent protein variants to changes in pH. *Biochemistry*. 38:5296–5301. <http://dx.doi.org/10.1021/bi9902182>
- Hajnóczky, G., L.D. Robb-Gaspers, M.B. Seitz, and A.P. Thomas. 1995. Decoding of cytosolic calcium oscillations in the mitochondria. *Cell*. 82:415–424. [http://dx.doi.org/10.1016/0092-8674\(95\)90430-1](http://dx.doi.org/10.1016/0092-8674(95)90430-1)
- Hanson, G.T., T.B. McAnaney, E.S. Park, M.E. Rendell, D.K. Yarbrough, S. Chu, L. Xi, S.G. Boxer, M.H. Montrose, and S.J. Remington. 2002. Green fluorescent protein variants as ratiometric dual emission pH sensors. I. Structural characterization and preliminary application. *Biochemistry*. 41:15477–15488. <http://dx.doi.org/10.1021/bi026609p>
- Hattori, T., K. Watanabe, Y. Uechi, H. Yoshioka, and Y. Ohta. 2005. Repetitive transient depolarizations of the inner mitochondrial membrane induced by proton pumping. *Biophys. J.* 88:2340–2349. <http://dx.doi.org/10.1529/biophysj.104.041483>
- Hoek, J.B., D.G. Nicholls, and J.R. Williamson. 1980. Determination of the mitochondrial protonmotive force in isolated hepatocytes. *J. Biol. Chem.* 255:1458–1464.
- Huang, Z., W. Zhang, H. Fang, M. Zheng, X. Wang, J. Xu, H. Cheng, G. Gong, W. Wang, R.T. Dirksen, and S.S. Sheu. 2011. Response to “A critical evaluation of cpYFP as a probe for superoxide.” *Free Radic. Biol. Med.* 51:1937–1940. <http://dx.doi.org/10.1016/j.freeradbiomed.2011.08.024>
- Hüser, J., and L.A. Blatter. 1999. Fluctuations in mitochondrial membrane potential caused by repetitive gating of the permeability transition pore. *Biochem. J.* 343:311–317. <http://dx.doi.org/10.1042/0264-6021.3430311>
- Hüser, J., C.E. Rechenmacher, and L.A. Blatter. 1998. Imaging the permeability pore transition in single mitochondria. *Biophys. J.* 74:2129–2137. [http://dx.doi.org/10.1016/S0006-3495\(98\)77920-2](http://dx.doi.org/10.1016/S0006-3495(98)77920-2)
- Iwata, S., J.W. Lee, K. Okada, J.K. Lee, M. Iwata, B. Rasmussen, T.A. Link, S. Ramaswamy, and B.K. Jap. 1998. Complete structure of the 11-subunit bovine mitochondrial cytochrome bc₁ complex. *Science*. 281:64–71. <http://dx.doi.org/10.1126/science.281.5373.64>
- Jach, G., M. Pesch, K. Richter, S. Frings, and J.F. Uhrig. 2006. An improved mRFP1 adds red to bimolecular fluorescence complementation. *Nat. Methods*. 3:597–600. <http://dx.doi.org/10.1038/nmeth901>
- Jacobson, J., and M.R. Duchen. 2002. Mitochondrial oxidative stress and cell death in astrocytes—requirement for stored Ca²⁺ and sustained opening of the permeability transition pore. *J. Cell Sci.* 115:1175–1188.
- Jiang, D., L. Zhao, and D.E. Clapham. 2009. Genome-wide RNAi screen identifies Letm1 as a mitochondrial Ca²⁺/H⁺ antiporter. *Science*. 326:144–147. <http://dx.doi.org/10.1126/science.1175145>
- Johnson, D.E., H.W. Ai, P. Wong, J.D. Young, R.E. Campbell, and J.R. Casey. 2009. Red fluorescent protein pH biosensor to detect concentrative nucleoside transport. *J. Biol. Chem.* 284:20499–20511. <http://dx.doi.org/10.1074/jbc.M109.019042>
- Klingenberg, M. 2008. The ADP and ATP transport in mitochondria and its carrier. *Biochim. Biophys. Acta*. 1778:1978–2021. <http://dx.doi.org/10.1016/j.bbamem.2008.04.011>
- Kneen, M., J. Farinas, Y. Li, and A.S. Verkman. 1998. Green fluorescent protein as a noninvasive intracellular pH indicator. *Biophys. J.* 74:1591–1599. [http://dx.doi.org/10.1016/S0006-3495\(98\)77870-1](http://dx.doi.org/10.1016/S0006-3495(98)77870-1)
- Krämer, R., and M. Klingenberg. 1980. Modulation of the reconstituted adenine nucleotide exchange by membrane potential. *Biochemistry*. 19:556–560. <http://dx.doi.org/10.1021/bi00544a025>
- Kroemer, G., L. Galluzzi, and C. Brenner. 2007. Mitochondrial membrane permeabilization in cell death. *Physiol. Rev.* 87:99–163. <http://dx.doi.org/10.1152/physrev.00013.2006>
- Lambert, A.J., and M.D. Brand. 2004. Superoxide production by NADH:ubiquinone oxidoreductase (complex I) depends on the pH gradient across the mitochondrial inner membrane. *Biochem. J.* 382:511–517. <http://dx.doi.org/10.1042/BJ20040485>
- Lemasters, J.J., E. Chacon, H. Ohata, I.S. Harper, A.L. Nieminen, S.A. Tesfai, and B. Herman. 1995. Measurement of electrical potential, pH, and free calcium ion concentration in mitochondria of living cells by laser scanning confocal microscopy. *Methods Enzymol.* 260:428–444. [http://dx.doi.org/10.1016/0076-6879\(95\)60156-2](http://dx.doi.org/10.1016/0076-6879(95)60156-2)
- Llopis, J., J.M. McCaffery, A. Miyawaki, M.G. Farquhar, and R.Y. Tsien. 1998. Measurement of cytosolic, mitochondrial, and Golgi pH in single living cells with green fluorescent proteins. *Proc. Natl. Acad. Sci. USA*. 95:6803–6808. <http://dx.doi.org/10.1073/pnas.95.12.6803>
- Ma, Q., H. Fang, W. Shang, L. Liu, Z. Xu, T. Ye, X. Wang, M. Zheng, Q. Chen, and H. Cheng. 2011. Superoxide flashes: early mitochondrial signals for oxidative stress-induced apoptosis. *J. Biol. Chem.* 286:27573–27581. <http://dx.doi.org/10.1074/jbc.M111.241794>
- Maloff, B.L., S.P. Scordilis, and H. Tedeschi. 1977. Membrane potential of mitochondrial measured with microelectrodes. *Science*. 195:898–900. <http://dx.doi.org/10.1126/science.841317>
- Martin, J., K. Mahlke, and N. Pfanner. 1991. Role of an energized inner membrane in mitochondrial protein import. Delta psi drives the movement of presequences. *J. Biol. Chem.* 266:18051–18057.
- Matsuyama, S., J. Llopis, Q.L. Deveraux, R.Y. Tsien, and J.C. Reed. 2000. Changes in intramitochondrial and cytosolic pH: early events that modulate caspase activation during apoptosis. *Nat. Cell Biol.* 2:318–325. <http://dx.doi.org/10.1038/35014006>
- Miesenböck, G., D.A. De Angelis, and J.E. Rothman. 1998. Visualizing secretion and synaptic transmission with pH-sensitive green fluorescent proteins. *Nature*. 394:192–195. <http://dx.doi.org/10.1038/28190>
- Mitchell, P. 1961. Coupling of phosphorylation to electron and hydrogen transfer by a chemi-osmotic type of mechanism. *Nature*. 191:144–148. <http://dx.doi.org/10.1038/191144a0>
- Mitchell, P. 1975. Proton translocation mechanisms and energy transduction by adenosine triphosphatases: an answer to criticisms. *FEBS Lett.* 50:95–97. [http://dx.doi.org/10.1016/0014-5793\(75\)80465-0](http://dx.doi.org/10.1016/0014-5793(75)80465-0)
- Mitchell, P., and J. Moyle. 1969. Estimation of membrane potential and pH difference across the cristae membrane of rat liver mitochondria. *Eur. J. Biochem.* 7:471–484. <http://dx.doi.org/10.1111/j.1432-1033.1969.tb19633.x>
- Muller, F.L. 2009. A critical evaluation of cpYFP as a probe for superoxide. *Free Radic. Biol. Med.* 47:1779–1780. <http://dx.doi.org/10.1016/j.freeradbiomed.2009.09.019>

- Nicholls, D.G. 1974. The influence of respiration and ATP hydrolysis on the proton-electrochemical gradient across the inner membrane of rat-liver mitochondria as determined by ion distribution. *Eur. J. Biochem.* 50:305–315. <http://dx.doi.org/10.1111/j.1432-1033.1974.tb03899.x>
- Nicholls, D.G. 2005. Commentary on: 'old and new data, new issues: the mitochondrial Deltapsi' by H. Tedeschi. *Biochim. Biophys. Acta.* 1710:63–65. <http://dx.doi.org/10.1016/j.bbabi.2005.09.002>
- Nowikovsky, K., E.M. Froschauer, G. Zsurka, J. Samaj, S. Reipert, M. Kolisek, G. Wiesenberger, and R.J. Schweyen. 2004. The LETM1/YOL027 gene family encodes a factor of the mitochondrial K⁺ homeostasis with a potential role in the Wolf-Hirschhorn syndrome. *J. Biol. Chem.* 279:30307–30315. <http://dx.doi.org/10.1074/jbc.M403607200>
- Palmieri, F. 2004. The mitochondrial transporter family (SLC25): physiological and pathological implications. *Pflugers Arch.* 447: 689–709. <http://dx.doi.org/10.1007/s00424-003-1099-7>
- Poburko, D., J. Santo-Domingo, and N. Demareux. 2011. Dynamic regulation of the mitochondrial proton gradient during cytosolic calcium elevations. *J. Biol. Chem.* 286:11672–11684. <http://dx.doi.org/10.1074/jbc.M110.159962>
- Porcelli, A.M., A. Ghelli, C. Zanna, P. Pinton, R. Rizzuto, and M. Rugolo. 2005. pH difference across the outer mitochondrial membrane measured with a green fluorescent protein mutant. *Biochem. Biophys. Res. Commun.* 326:799–804. <http://dx.doi.org/10.1016/j.bbrc.2004.11.105>
- Pouvreau, S. 2010. Superoxide flashes in mouse skeletal muscle are produced by discrete arrays of active mitochondria operating coherently. *PLoS ONE.* 5:e13035. <http://dx.doi.org/10.1371/journal.pone.0013035>
- Rottenberg, H. 1975. The measurement of transmembrane electrochemical proton gradients. *J. Bioenerg.* 7:61–74. <http://dx.doi.org/10.1007/BF01558427>
- Saraste, M. 1999. Oxidative phosphorylation at the fin de siècle. *Science.* 283:1488–1493. <http://dx.doi.org/10.1126/science.283.5407.1488>
- Schwarzländer, M., D.C. Logan, M.D. Fricker, and L.J. Sweetlove. 2011. The circularly permuted yellow fluorescent protein cpYFP that has been used as a superoxide probe is highly responsive to pH but not superoxide in mitochondria: implications for the existence of superoxide 'flashes'. *Biochem. J.* 437:381–387. <http://dx.doi.org/10.1042/BJ20110883>
- Scorrano, L., M. Ashiya, K. Buttle, S. Weiler, S.A. Oakes, C.A. Mannella, and S.J. Korsmeyer. 2002. A distinct pathway remodels mitochondrial cristae and mobilizes cytochrome c during apoptosis. *Dev. Cell.* 2:55–67. [http://dx.doi.org/10.1016/S1534-5807\(01\)00116-2](http://dx.doi.org/10.1016/S1534-5807(01)00116-2)
- Shaner, N.C., M.Z. Lin, M.R. McKeown, P.A. Steinbach, K.L. Hazelwood, M.W. Davidson, and R.Y. Tsien. 2008. Improving the photostability of bright monomeric orange and red fluorescent proteins. *Nat. Methods.* 5:545–551. <http://dx.doi.org/10.1038/nmeth.1209>
- Strauss, M., G. Hofhaus, R.R. Schröder, and W. Kühlbrandt. 2008. Dimer ribbons of ATP synthase shape the inner mitochondrial membrane. *EMBO J.* 27:1154–1160. <http://dx.doi.org/10.1038/emboj.2008.35>
- Talbot, J., J.N. Barrett, E.F. Barrett, and G. David. 2007. Stimulation-induced changes in NADH fluorescence and mitochondrial membrane potential in lizard motor nerve terminals. *J. Physiol.* 579:783–798. <http://dx.doi.org/10.1113/jphysiol.2006.126383>
- Tantama, M., Y.P. Hung, and G. Yellen. 2011. Imaging intracellular pH in live cells with a genetically encoded red fluorescent protein sensor. *J. Am. Chem. Soc.* 133:10034–10037. <http://dx.doi.org/10.1021/ja202902d>
- Thiffault, C., and J.P. Bennett Jr. 2005. Cyclical mitochondrial deltapSiM fluctuations linked to electron transport, F0F1 ATP-synthase and mitochondrial Na⁺/Ca²⁺ exchange are reduced in Alzheimer's disease cybrids. *Mitochondrion.* 5:109–119. <http://dx.doi.org/10.1016/j.mito.2004.12.002>
- Vergun, O., and I.J. Reynolds. 2004. Fluctuations in mitochondrial membrane potential in single isolated brain mitochondria: modulation by adenine nucleotides and Ca²⁺. *Biophys. J.* 87:3585–3593. <http://dx.doi.org/10.1529/biophysj.104.042671>
- Vergun, O., T.V. Votyakova, and I.J. Reynolds. 2003. Spontaneous changes in mitochondrial membrane potential in single isolated brain mitochondria. *Biophys. J.* 85:3358–3366. [http://dx.doi.org/10.1016/S0006-3495\(03\)74755-9](http://dx.doi.org/10.1016/S0006-3495(03)74755-9)
- Wang, W., H. Fang, L. Groom, A. Cheng, W. Zhang, J. Liu, X. Wang, K. Li, P. Han, M. Zheng, et al. 2008. Superoxide flashes in single mitochondria. *Cell.* 134:279–290. <http://dx.doi.org/10.1016/j.cell.2008.06.017>
- Wiederkehr, A. 2009. Matrix alkalisation unleashes β -cell mitochondria. *Islets.* 1:154–156. <http://dx.doi.org/10.4161/isl.1.2.9058>
- Wiederkehr, A., K.S. Park, O. Dupont, N. Demareux, T. Pozzan, G.W. Cline, and C.B. Wollheim. 2009. Matrix alkalization: a novel mitochondrial signal for sustained pancreatic beta-cell activation. *EMBO J.* 28:417–428. <http://dx.doi.org/10.1038/emboj.2008.302>
- Zorov, D.B., C.R. Filburn, L.O. Klotz, J.L. Zweier, and S.J. Sollott. 2000. Reactive oxygen species (ROS)-induced ROS release: a new phenomenon accompanying induction of the mitochondrial permeability transition in cardiac myocytes. *J. Exp. Med.* 192:1001–1014. <http://dx.doi.org/10.1084/jem.192.7.1001>
- Zotova, L., M. Aleschko, G. Sponder, R. Baumgartner, S. Reipert, M. Prinz, R.J. Schweyen, and K. Nowikovsky. 2010. Novel components of an active mitochondrial K⁽⁺⁾/H⁽⁺⁾ exchange. *J. Biol. Chem.* 285:14399–14414. <http://dx.doi.org/10.1074/jbc.M109.059956>