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Review

Shedding light on mitochondrial outer-membrane permeabilization and membrane potential: State of the art methods and biosensors

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ABSTRACT

Membrane structural integrity is essential for optimal mitochondrial function. These organelles produce the energy needed for all vital processes, provided their outer and inner membranes are intact. This prevents the release of mitochondrial apoptogenic factors into the cytosol and ensures intact mitochondrial membrane potential ($\Delta\Psi_m$) to sustain ATP production. Cell death by apoptosis is generally triggered by outer mitochondrial membrane permeabilization (MOMP), tightly coupled with loss of $\Delta\Psi_m$. As these two processes are essential for both mitochondrial function and cell death, researchers have devised various techniques to assess them. Here, we discuss current methods and biosensors available for detecting MOMP and measuring $\Delta\Psi_m$, focusing on their advantages and limitations and discuss what new imaging tools are needed to improve our knowledge of mitochondrial function.

1. Introduction

Mitochondria are organelles involved in life and death, as they are both the cellular powerhouse and the dynamite that can trigger a programmed and tightly regulated process called apoptosis. While their role in bioenergetics was recently widely covered [1] [2], this review addresses the central role mitochondria play during apoptosis. More specifically, we will focus on recent methods and fluorescent biosensors to detect, visualize and quantify mitochondrial outer-membrane permeabilization (MOMP) and mitochondrial membrane potential ($\Delta\Psi_m$) that are indissociable from one another [3]. $\Delta\Psi_m$ is an electrochemical gradient of approximately -180 mV established across the inner mitochondrial membrane (IMM), with the matrix negative compared to the intermembrane space. $\Delta\Psi_m$ is a direct result of the mitochondrial membrane composition. While the outer mitochondrial membrane (OMM) has a certain degree of porosity and it allows the passage of ions and small molecules, the IMM has no porosity. Therefore, ions and larger molecules are all transported through specific transporter proteins, each specific to a certain type of ion. As a consequence of this selectivity, an ion gradient is established across the IMM and the H^+ gradient is used by the oxidative phosphorylation (OXPHOS) complexes for ATP production

from ADP and inorganic phosphate (P_i) (reviewed in [4]).

MOMP is widely accepted as a “point-of-no-return” during apoptosis as it allows the cytosolic release from the intermembrane space (IMS) of several apoptogenic proteins such as cytochrome *c*. This occurs through rupture of the OMM, following activation of the pore-forming BCL2 proteins BAX, BAK, BOK and the recently described truncated BID [5]. Once released into the cytosol, cytochrome *c* assembles with APAF1 monomers into a caspase-activating platform called the apoptosome, leading to a rapid chain reaction activation of caspases 9, 3 and 7 [6]. These proteases then cleave hundreds of vital cellular substrates, leading to fast and efficient cell death. Interestingly, loss of OMM integrity is concomitant to a sudden decrease in $\Delta\Psi_m$ and a subsequent loss of its ATP producing capacity. Thus, even if caspase activation is inhibited, cells still die through caspase-independent cell death [7].

The long-standing dogma was that MOMP occurred in all mitochondria in a cell, in a fast and concerted manner, inducing destructive downstream caspase activation. However, this is undermined by studies describing that caspases could be activated in a non-lethal context, and thus play non-apoptotic roles in various settings, ranging from cancer development, immune cells maturation and neuronal function or myogenic differentiation [8] [9]. These observations were substantiated

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by recent advances in high-resolution microscopy and fluorescent biosensors revealing how mitochondria act during apoptosis, and which highlighted that sub-lethal apoptotic stresses could lead to the permeabilization of only few mitochondria and subsequently to non-lethal caspase activation [10] [11]. Aside from its major role in triggering caspase activation, MOMP also participates in inflammation, through the activation of cGAS/STING and NF κ B pathways [12] [13] [14] [15]. These discoveries were made following the development of various techniques and biosensors allowing the detection and visualization of MOMP. It is therefore paramount to summarize them in a chronological manner and critically assess their advantages and limitations, and what technical advances are still needed in order to shed light on such an important cellular process.

2. Methods and biosensors for detecting and quantifying MOMP

2.1. Fractionation and biochemistry methods

Seminal studies assessing MOMP mainly relied on screening the presence in the cytosol of mitochondrial cytochrome *c* or other proteins of the IMS. This is based on mechanical or detergent-based cytosolic fractionation methods, using Dounce or Potter-Elvehjem homogenizers or digitonin. These fractionation methods were successfully used to first describe MOMP and identify the apoptogenic proteins that are released from the IMS. In 1996, the team of Xiaodong Wang used mechanical cytosolic fractionation to identify, for the first time, the mitochondrial release of cytochrome *c* as a trigger for apoptosis. This was followed by the team of Douglas Green who employed the same technique to demonstrate how BCL-2 family proteins regulate MOMP [16] [17] [18], including the mitochondrial translocation of BAX, [19–22] and subsequent OMM perforation during apoptosis [23] [22]. These fractionation methods have a clear advantage, as they are easy to establish, yet they have a low throughput and can pose reproducibility issues, as mechanical plasma membrane rupture is often dependent on the individual conducting the experiment. Moreover, detergent concentrations required to solely permeabilize the plasma membrane need to be determined for each cell line, because longer incubation times could lead to unwanted OMM permeabilization, especially since pre-apoptotic fragmented mitochondria are more sensitive to damage compared to non-apoptotic organelles [24].

Digitonin-mediated plasma membrane permeabilization at very low concentrations was later combined with intramitochondrial immunostaining of cytochrome *c* to distinguish apoptotic from viable cells using flow cytometry analysis. In apoptotic cells, MOMP and digitonin treatment lead to the loss of both mitochondrial and cytosolic cytochrome *c* pools, whereas viable cells retain cytochrome *c* within the IMS [25] [26]. This method relies on the difference in cholesterol content between the plasma and mitochondrial membranes: as the latter are very poor in cholesterol, digitonin selectively permeabilizes the cholesterol-rich plasma membrane, allowing the washout of cytosolic cytochrome *c* [27]. Following cytochrome *c* immunostaining, viable cells display a bright staining, while apoptotic cells have a distinct, low-intensity signal. This technique is relatively fast compared to classical Western blotting and immunofluorescence techniques that can take days, and allows the assessment of MOMP in hundreds of thousands of cells treated with various apoptotic inducers [26].

2.2. Mitochondrial potentiometric dyes

Measuring the activity of mitochondria is a long-standing need in the community, which has been seeking an user-friendly and widely implementable solution, and turned to rhodamine esters. These red fluorescent probes are cationic and lipophilic, two key features that allow them to easily cross membranes and accumulate in negatively-charged compartments such as mitochondria [28]. Therefore, they are convenient tools to respond to variations in $\Delta\Psi_m$, which is used as a

readout of mitochondrial activity and therefore as a proxy for MOMP since $\Delta\Psi_m$ is usually lost following MOMP. The current most extensively used rhodamine derivatives are tetramethylrhodamine ethyl and methyl esters – TMRE and TMRM, respectively. Originally engineered and validated in the late 1980 s and early 1990 s, these cationic dyes are convenient due to their small size, high signal-to-noise ratio, and low cytotoxicity [29,30]. Furthermore, both dyes easily cross the plasma membrane, and they are readily and reversibly incorporated in active mitochondria in a voltage-dependent manner. In this context, they diffuse across the mitochondrial membrane following the Nernst equation, and accumulate in the organelle matrix which is negatively charged [29]. Of note, the concentration of the probe is a critical parameter to correctly assess mitochondrial membrane potential. At concentrations below 150 nM, TMRM was shown to have negligible effects on OXPHOS in isolated mitochondria, while TMRE impaired mitochondrial respiration [28]. However, it should also be noted that TMRM fluorescence undergoes quenching and a red shift in excitation and emission spectra at concentrations higher than 30–50 nM in cultured cells [28] [31] [32]. The TMRM quenching mode is described in more detail in the paragraphs below. TMRM also displays a lower membrane-bound fraction than TMRE, strongly supporting the fact that fluctuations in TMRM fluorescence – but not in TMRE – are almost exclusively correlated with alterations of the mitochondrial membrane potential [28]. Therefore, TMRM is to date one of the most common strategies to analyze $\Delta\Psi_m$ variations, such as the one occurring following widespread MOMP.

Initial studies also revealed that TMRM fluorescence may be altered in a $\Delta\Psi_m$ -independent manner by changes in mitochondrial protein concentration [28]. Therefore, these observations raised the need for implementing strategies to normalize TMRM fluorescence over that of an alternative fluorescent marker impervious to $\Delta\Psi_m$ variations. In line with this, MitoTracker probes were designed and constitute the most common fluorescent dyes used as mitochondrial markers, with some of them being sensitive to $\Delta\Psi_m$ changes [33]. Although their use is widespread, these dyes can be toxic in specific cellular contexts [34], and their output should be properly interpreted [35]. Among them, MitoTracker Green is the only MitoTracker probe to retain its fluorescence when the $\Delta\Psi_m$ is dissipated owing to its covalent binding to thiols in mitochondria [33] [35]. Conversely to MitoTracker DeepRed, the fluorescence of which decreases when $\Delta\Psi_m$ is lost [35], MitoTracker Green is suitable to measure mitochondrial mass irrespective of mitochondrial membrane potential. Therefore, the TMRM/MitoTracker Green ratio seems to be an adequate solution to overcome TMRM variations due to changes in mitochondrial protein concentrations [36], while TMRM/MitoTracker Deep Red analyses could be prone to creating $\Delta\Psi_m$ -related artifacts. Alternatively, TMRM fluorescence can be normalized with genetically-encoded fluorescent proteins targeted to mitochondria *i.e.*, mito-GFP, mito-mCherry, *etc.* While it is worth mentioning that conditions which favor mitochondrial acidification may lead to GFP quenching [37], the use of mitochondria-targeted fluorescent proteins seems to be a pertinent alternative to the use of MitoTracker probes for TMRM normalization [38].

As introduced before, the normalization of the TMRM fluorescent signal is mandatory to correctly estimate $\Delta\Psi_m$. However, it should be kept in mind that such normalization makes of TMRM a relative measure of $\Delta\Psi_m$ and not an absolute one. These considerations also imply the understanding of the chemical features of TMRM, such as its “quenched mode”. If the concentration of the probe is too high (generally above 50 nM although this value is cell-specific and/or condition-specific and must be determined experimentally), fluorescent TMRM molecules are quenched in the matrix. The incubation with the protonophore Carbonyl cyanide 3-chlorophenylhydrazone (CCCP), which should normally make $\Delta\Psi_m$ collapse, induces a transient peak in TMRM fluorescence, eventually followed by loss of TMRM signal [39]. This is known as the “quenched mode”, and occurs when the probe leaves the matrix and becomes unquenched, thereby leading to an overall increase in the

signal. Conversely, this peak will be undetectable if TMRM is used in a “non-quenched” mode (*i.e.*, at a concentration below the quenching point), and the probe retains its fluorescence in the organelle matrix. Under these conditions, incubating the samples with CCCP will induce $\Delta\Psi_m$ depolarization and with this, a significant loss in TMRM fluorescence. While TMRM is often used in non-quenched mode, using TMRM in quenched mode can be useful when one seeks to measure fast, transient variations in $\Delta\Psi_m$ in a specific experimental condition. However, this mode shouldn't be used to compare $\Delta\Psi_m$ differences among populations or conditions. Last, it is worth mentioning that TMRM can accumulate in the plasma membrane because of its sensitivity to the negative potential of approximately -60 mV and known as $\Delta\Psi_p$ (plasma membrane potential, as opposed to $\Delta\Psi_m$ for mitochondrial membrane potential). Therefore, it must be considered that $\Delta\Psi_p$ may also contribute to the overall fluorescence intensity of the dye [40]. Although this contribution is limited when TMRM is used in a “non-quenched” mode due to the slow accumulation of the probe across the plasma membrane, it should not be considered as negligible. Therefore, best practices would include an estimation of $\Delta\Psi_p$ along with $\Delta\Psi_m$, using fluorescent probes such as Bis-(1,3-Dibutylbarbituric Acid)Trimethine Oxonol/DiBAC₄(3) [39] [41] [42] [43,44].

Given their flexibility and sensitivity towards $\Delta\Psi_m$ in a variety of biological paradigms, TMRM and TMRE emerged as interesting tools to study the consequences of MOMP. A significant decrease in TMRM or TMRE fluorescence was observed rapidly after cytochrome *c* release from mitochondria, soon after the incubation of cultured cancer cells with apoptosis-inducing pharmacological treatments [3] [25] [45]. This led to the conclusion that $\Delta\Psi_m$ loss is an early feature of paradigms in which MOMP is activated. To date, TMRM is still employed as a bona-fide reporter of early MOMP activation in order to dissect its temporal kinetics in single cells, as well as to identify the proteins involved in this signaling pathway [45] [46] [47] [48]. It can also be used in conjunction with other fluorescent proteins, such as genetically-encoded Förster's Resonance Energy Transfer (FRET) donor-acceptor pairs to assess BAX oligomerization [46]. Interestingly, TMRM fluorescence kinetics were integrated with that of five fluorescent dyes in a high-throughput screening to determine apoptosis onset and progression [48]. Last, TMRE and TMRM also helped to identify mitoflashes/mitoflickers, transient mitochondrial depolarizations correlating with mPTP opening and ROS-based signaling occurring in physiological conditions [49] [50] [51].

As an alternative to TMRM and TMRE, which are monochromatic probes, a two-color dye called 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzamidozolocarbocyanin iodide (JC-1) is sensitive to $\Delta\Psi_m$ and has also been used to investigate MOMP because of its cationic and lipophilic properties. The emission spectrum of JC-1 shifts from green (λ_{em} 530 nm) to red (λ_{em} 590 nm) upon $\Delta\Psi_m$ loss, along with the aggregation of the red form of the probe in structures known as J-aggregates [52]. This change in the fluorescent emission spectra and aggregation features of JC-1 are observed when $\Delta\Psi_m$ raises above $\sim 80/100$ mV, and both the aggregation and spectral shift features of the probe were shown to be reversible if mitochondria restore a canonical $\Delta\Psi_m$ [53].

Although the presence of red and green species should allow the discrimination of organelles that are polarized and those that are not, JC-1 was shown to have several caveats. First and foremost, fluorescence variations of the green, monomeric form of the dye were shown to better showcase $\Delta\Psi_m$ variations than those of the red, aggregated form. This is not a general principle, but variations in the red form of JC-1 were also shown to occur in specific experimental conditions, such as after H₂O₂ treatment in neuronal cells, and they appear to be independent of $\Delta\Psi_m$ [54,55]. Second, the presence or absence of JC-1 aggregates should allow to determine the polarization status of mitochondria in a rapid, visual fashion. However, subtle variations in $\Delta\Psi_m$ are much harder to detect, and it was reported that the sensitivity of JC-1 is rather limited in conditions where the alteration of mitochondrial membrane potential is small [56]. Third, the two fluorescent species accumulate differently,

with the green species equilibrating ~ 6 times faster than the red dye in cardiomyocytes [57]. In these cells, the red species were reported to disappear in cells incubated with carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone (FCCP), a mitochondrial uncoupler similar to CCCP. One possibility is that since the equilibration time depends on the surface-to-volume ratio, JC-1 may display $\Delta\Psi_m$ -independent differences in cell types or subcellular locations where the surface-to-volume ratio is heterogeneous [56]. Fourth, JC-1 is capable of producing J-aggregates only in a concentration-dependent manner. Due to increased IMM permeability upon $\Delta\Psi_m$ loss, the incorporation of the green form of JC-1 into depolarized mitochondria is less efficient and it may not reach the required concentration to produce JC-1 aggregates [53]. Last, JC-1 was also shown to be toxic and sensitive to loading concentrations, with the green species partially disappearing when JC-1 was used in saturated conditions [56,57]. Nonetheless, JC-1 can still be used to assess wide $\Delta\Psi_m$ variations during MOMP, for example during BH3 profiling which is a technique testing apoptotic priming and that relies on the imaging of the red form of JC-1 [58].

For all these reasons, it would be necessary to calibrate the readout of JC-1 in each specific cell type of interest, and to verify that its variations correspond to real differences in $\Delta\Psi_m$. While this probe has already been used to follow MOMP [59,60], such careful validation of JC-1 behavior is experimentally cumbersome. Overall, it appears that the outcome of JC-1 is less generalized and easy-to-interpret than the one of TMRM/TMRE. For all these reasons, it would be preferable to avoid using JC-1 to estimate $\Delta\Psi_m$.

2.3. Intermembrane space fluorescent proteins (IMS-FP) probes

Monitoring MOMP can also be done by visualizing the release through OMM pores of IMS proteins fused to fluorescent tags. One of the first studies using cytochrome *c*-GFP-expressing cells deciphered the cascade reactions during apoptosis [61]. In cells undergoing complete MOMP, the authors observed rapid and simultaneous release of cytochrome *c* from all mitochondria, long before other signs of apoptosis such as phosphatidyl serine exposure and loss of cell membrane integrity were seen, establishing the mitochondrial release of cytochrome *c* as a reliable marker for early apoptosis [61]. In a similar way, researchers established the localization in the IMS and the apoptogenic release following MOMP of several other proteins such as AIF, EndoG or Omi/HtraA2 [62–64] [65]. While extending our knowledge on MOMP, this approach could be biased by the choice of the fusion protein as it is now well accepted that different IMS soluble proteins are released into the cytosol with different kinetics [66]. To overcome this, one could use CRISPR/Cas9 gene editing to endogenously tag IMS proteins with different fluorescent tags and simultaneously assess their mitochondrial release [67]. Moreover, although these recombinant proteins allow MOMP visualization through loss of localized fluorescence signal within the IMS, they do not allow the tracking of permeabilized mitochondria. Another limitation is the detection limit imposed by the half-life of IMS proteins once released into the cytosol. For example, cytochrome *c* can be rapidly degraded in neurons by the E3 ligase PARC/CUL9 upon MOMP, rendering its cytosolic detection problematic, especially in cells with high proteasomal activity [68].

2.4. Super-resolution nanoscopy

All the above-mentioned methods give a coarse overview of MOMP. The nature of OMM rupture leading to widespread MOMP was for a long time a matter of heated debate among cell biologists, some of them supporting the lipid pore model (the toroidal model) while others providing evidence for a protein model [69].

This argument was settled with the recent advances in microscopy. Cryo-electron microscopy studies in the early 2000 s on proteoliposomes loaded with physiological levels of OMM proteins (called outer membrane vesicles or OMVs) revealed pore-like structures formed

by activated BAX. These pores with diameters between 25 and 100 nm were suggested to be lipidic and not proteinaceous, as pore edges were smooth, likely due to the imaging technique, which could be applied to small liposomes but not whole mitochondria [70] [71] [69]. Visualization of OMM pores was later facilitated by super-resolution nanoscopy such as stimulated emission depletion microscopy (STED) and single-molecule localization microscopy (SMLM), providing image resolutions in the range of 60 and 20 nm, respectively. In 2016, the teams of both Stefan Jakobs and Ana García-Sáez simultaneously shed a new resolute light on MOMP by using mitochondria in live cells experiencing MOMP and not artificial liposomes. Große and colleagues imaged cells undergoing actinomycin D-induced MOMP using STED microscopy and revealed BAX assembly into ring-like structures (BAX rings) permitting cytochrome *c* release. BAX rings appeared smaller than 400 nm in diameter, similar to the pore size estimated by previous cryo-EM studies [72].

These findings were complemented by Salvador-Gallego et al., that tackled OMM pore architecture with a plethora of imaging techniques such as SMLM, STED and atomic force microscopy. They succeeded in visualizing and measuring BAX rings and arc-shaped assemblies, both being able to pierce the OMM. These super-resolution microscopy techniques support a model whereby single molecules of activated BAX are gradually inserted into the OMM, then self-assemble into larger supramolecular lines and arc structures until they are able to reduce the lipid curvature tension and create pores in the OMM. Some of these pores have edges fully decorated with BAX rings, while others have arcs of BAX, yet both types of structures are able to perforate the OMM [73]. Using the same imaging techniques, García-Sáez's team resolved the structure of BOK pores and extended the BCL2 pore-forming proteins to truncated BID that can act as a BAK-like MOMP effector [74] [5]. Similar high-resolution imaging techniques were also successfully used to visualize the release of mitochondrial DNA following mitochondrial outer and inner membrane permeabilization [75,76].

While the super-resolution images of BAX pores were impressive and made history in the apoptosis research community, they are nonetheless limited as they provide only snapshots of a dynamic process. Future research should focus on the development of live super-resolution imaging for permeabilizing mitochondria to establish whether BAX assemblies into clearly distinct structures or whether they are interconvertible in time, with the lines growing into arcs and then gradually becoming rings. Lastly, the super-resolution image definition of BAX rings would greatly benefit from fine biochemistry analysis if future techniques allow the specific isolation of these structures from permeabilized mitochondria.

2.5. MOMP biosensors

Historically, MOMP was considered to be an all-or-nothing event, with all cytochrome *c* being suddenly released from all mitochondria within a short time frame, leading to massive and lethal caspase activation. Yet, this did not reflect growing evidence that caspases can be non-lethally activated and play vital roles in both normal organismal development and cancer [77] [8] [78]. This knowledge gap was addressed by the development of sensitive fluorescent biosensors which allowed the visualization of MOMP in only a minority of mitochondria (hereafter called minority MOMP).

While insufficient to induce apoptosis, minority MOMP is responsible for triggering low-level caspase activation, subsequent DNA damage as a consequence of CAD DNase derepression, and ultimately oncogenic transformation [11] [78]. To mark and quantify single permeabilized mitochondria, Stephen Tait's team designed a two-probe biosensor: a cytosolic FKBP-GFP probe (cytoGFP) and an IMM-anchored mCherry-tagged FRB fusion protein (mitoCherry). This biosensor relies on the chemically dimerizable FK506 Binding Protein (FKBP) and FKBP-Rapamycin Binding domain (FRB) protein domains [79] [80]. In cells with undamaged mitochondria the two probes are

differentially compartmentalized within the cytosol and IMS, whereas during MOMP the cytoGFP enters the IMS through OMM pores and stably binds the mitoCherry probe, in the presence of the chemical heterodimerizer [11] [24]. Using this approach, the researchers found that sub-lethal stresses such as BH3 mimetics, proteasome inhibitors, or low expression of BH3-only proteins can induce minority MOMP and promote oncogenic transformation. Moreover, this phenomenon is enhanced by mitochondrial fission, which prevents homogenous distribution of anti-apoptotic BCL2 proteins throughout the mitochondrial network [24]. More physiologically, a recent study identified minority MOMP within individual axonal mitochondria, which was correlated with localized non-apoptotic caspase activation [81]. These findings are also in line with previous studies showing that neurons can withstand MOMP in up to 15% of their total mitochondrial content [82].

While extremely useful in identifying single permeabilized mitochondria in a non-apoptotic context, quantifying minority MOMP using the cytoGFP/mitoCherry probes is time-consuming and not amenable to high-throughput quantitative microscopy in its current form. In 2022, Green's team improved minority MOMP detection and quantification by proposing a new biosensor based on split superfolder GFP (sfGFP). More precisely, the first 10 beta-strands of sfGFP (GFP1–10) were fused to mCherry and anchored in the IMM, while the 11th strand (GFP11) is localized in the cytosol, with no fluorescence being detected [10] [83] [84]. When MOMP occurs, GFP11 diffuses into the IMS, the 11 beta-strands complement and emit the green fluorescent signal. A clear advantage of this biosensor is its compatibility with flow cytometry analysis and cell sorting, thus enabling more detailed biochemistry and transcriptomic analyses [10]. Using this biosensor, the team elegantly showed that minority MOMP is required for the survival of drug-tolerant persister cancer cells that are most likely responsible for lethal cancer recurrence [10].

2.6. *In vivo* MOMP detection

Most current data on MOMP arose from *in vitro* studies. To better understand how cell death shapes both developmental processes and pathologies like cancer, *in vivo* visualization is essential, ideally at a sub-cellular resolution. Unfortunately, there are no major advancements in detecting MOMP *in vivo* in the last years, probably due to focusing on rather imaging and quantifying lethal caspase activation and finally cell death, with MOMP considered a marginal event.

A proof-of-concept study showing that visualizing MOMP *in vivo* is achievable came from the Weissleder's team in 2012 [85]. The researchers stably expressed soluble mitochondrial intermembrane space fluorescence reporter proteins (IMS-RP consisting of SMAC¹⁻⁵⁹ fused with either GFP or mCherry) in breast or pancreatic cancer cells that were then grafted in nude mice and developed into tumors [85]. They then successfully imaged the mitochondrial release of IMS-RP through window chambers, and temporally assessed the occurrence of MOMP in tumors treated with the pro-apoptotic BH3 mimetic drug ABT-263. This study thus showed the feasibility of imaging MOMP *in vivo*. The same team improved the intravital microscopy (IVM) method in 2015 by developing a DNA damage sensor [86].

The design of *in vivo* MOMP biosensors could also build upon the study of Orth and colleagues that used high-resolution IVM to determine *in vivo* the consequences of treating tumors with the antimetabolic drug paclitaxel [87]. The researchers grafted fibrosarcoma cells stably expressing H2B-mRFP and mEGFP-tubulin, allowing the visualization of chromatin and microtubules, respectively. When the tumors formed, the team was able to image through dorsal skinfold chambers the cellular response to paclitaxel and its single-cell pharmacodynamics: most cells enter a mitotic arrest, while some die through apoptosis [87]. The practicality of IVM for detecting cancer cells with activated effector caspases downstream of MOMP was also established by several other studies [88] [89]. Here, the scientists used FRET biosensors consisting of donor and acceptor fluorophores separated by a caspase cleavage site

and thus reporting caspase activation by a decrease in energy transfer towards the acceptor molecule. The above mentioned IVM methods are therefore very likely to be amenable to visualizing and quantifying MOMP, by including the IMS-RP biosensor for example.

3. What the future looks like for MOMP biosensors

Although well-characterized *in vitro*, there are still a number of questions related to triggering MOMP *in vivo*: does complete MOMP occur systematically in most cancer cells or are side-effects such as minority MOMP more common? What is the microenvironmental impact on the kinetics of MOMP, in terms of availability of growth factors and nutrients or angiogenesis? It still remains unclear what happens with permeabilized mitochondria, how they are trafficked, degraded or even if certain mitochondria eventually recover [90] [91]. For cancer treatment, what are the most effective drugs and treatment regimens to induce MOMP? In addition to gaining new mechanistic insights into how MOMP occurs *in vivo*, within tissues, organs or tumors, novel MOMP biosensors will also be revealing of the pharmacokinetics and pharmacodynamics of various drugs. This is critical for understanding and improving their anti-cancer efficacy.

These *in vivo* biosensors will need to meet critical requirements, such as deep tissue signal penetrance and high signal-to-noise ratio. Ideally, these imaging tools could be based on near infrared (NIR) fluorescent proteins that are compatible with deep-tissue, real-time, automated imaging and provide images that could be semi-automatically analyzed [92]. A research axis could focus on the use of the split superfolder NIR fluorescent protein-based MOMP biosensors, designed in a similar way to the minority MOMP reporter proposed by Green's lab [93] [94] [10]. To image and track the same cell(s) in consecutive imaging sessions over several days, these biosensors could be co-expressed with photo switchable fluorophores such as Dendra2 coupled to the histone 2B (H2B-Dendra2), to stably photomark the cells of interest [95] [89] [92].

As fluorescence-based biosensors require external illumination for fluorescence transfer which potentially produces a non-negligible amount of background noise, photobleaching and photocytotoxicity, alternative recombinant proteins based on bioluminescence resonance

energy transfer (BRET) could be proposed to follow MOMP *in vivo*. BRET imaging uses luciferase donors coupled with a fluorescent protein acceptor [96]. One promising luciferase is the NanoLuc (NLuc) that was isolated from deep sea shrimp *Oplophorus gracilirostris* [97]. NLuc is a small (19 kDa) protein with approximately 150-fold greater bioluminescence compared to the conventional Renilla luciferase. As for sfGFP, a binary split NLuc called NanoBiT has already been engineered and tested [98]. In this system, NLuc is divided into two subunits: an 18 kDa polypeptide called "Large BiT" and a 1.3 kDa peptide termed "Small BiT". The team of Antonio Amelio demonstrated that a BRET reporter based on NLuc fused to eGFP or to a large Stokes shift orange fluorescent protein (LSSmOrange), can be successfully used for *in vivo* spatiotemporal monitoring of small populations of tumor cells during mouse tumorigenesis [99]. Thus, one can propose a dual NanoBiT BRET sensor in which Large BiT could be targeted in the IMS and the Small BiT bound to eGFP or LSSmOrange allowing the visualization of mitochondria undergoing MOMP (Fig. 1A). An important challenge will also be to develop cell tracing genetic tools to follow *in vivo* and longitudinally characterize the fate of both normal and cancer cells that have undergone minority MOMP. One such tool could rely on IMS-targeted CRE recombinase, that would be released from mitochondria following minority MOMP and would excise the LoxP-Stop-LoxP cassette upstream of a genetically encoded fluorescent protein, thereby allowing its expression (Fig. 1B).

Once validated on xenografted tumors grown from cells stably expressing the biosensors, these tools could be genetically co-expressed in well-established genetic cancer mouse models such as the APC^{min/+} or MMTV-PyMT mice [100] [101]. Compared to classical cell xenografting models, these mice more accurately reflect/reproduce the stepwise oncogenic process. Aside from murine models, these biosensors could also be used in other species, such as zebrafish or chicken embryos, both models providing the advantage of having transparent embryos at early developmental stages, thus facilitating IVM [102].

4. Concluding remarks

Monitoring mitochondria during and after MOMP remains a

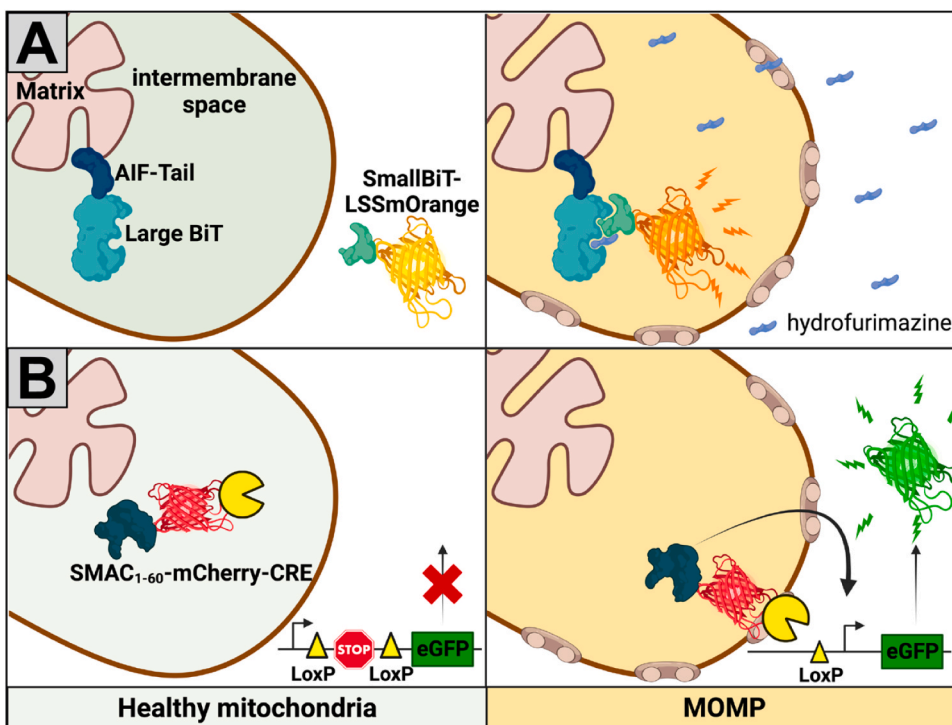


Fig. 1. Putative biosensors for monitoring MOMP *in vivo*. A. Molecular strategy based on a dual NanoBiT composed of IMS-localized Large BiT using an AIF-N-terminus sequence and a Small BiT-LSSmOrange recombinant protein. During MOMP, the SmallBiT-LSSmOrange fusion protein gains access to the IMS and NLuc is restructured. In the presence of its substrate, hydrofurimazine, NLuc emits light that will further activate the LSSmOrange moiety via BRET. B. Strategy for tracing cells undergoing minority MOMP. CRE recombinase is localised to the intermembrane space through fusion with SMAC₁₋₆₀ and mCherry for controlling correct localization. In cells undergoing MOMP and constitutively expressing a LoxP-STOP-LoxP eGFP transgene, CRE-mediated excision of the STOP cassette would allow eGFP expression and facilitate tracing cells surviving MOMP. Figure generated using BioRender.

challenging task. The initial use of robust biochemical protocols for describing the changes in the mitochondrial $\Delta\Psi_m$ and permeability during apoptosis paved the way for understanding the underlying molecular mechanisms. However, as MOMP can occur in relatively few mitochondria with various consequences, new and more sensitive chemical dyes and biosensors need to be designed and tested to follow, in real time and *in vivo*, the fate of cells undergoing minority MOMP. This will require the joined forces of chemists, molecular and cellular biologists. Eventually, these new tools should reveal the functional consequences of minority MOMP during physiological and pathological settings.

Declaration of Competing Interest

The authors declare that there is no conflict of interest.

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