# *In vivo* imaging of drug-induced mitochondrial outer membrane permeabilization at single cell resolution

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## Supplementary Information

### *Histology*

Human osteosarcoma HT1080 H2B-mCherry cells (ATCC) suspended in phosphate buffered saline (PBS) were mixed 1:1 with Matrigel (BD Biosciences), then injected sub-cutaneously into the flanks of a nude mouse (5e+06 cells per flank). After one week the tumors were approximately 5 mm in diameter. Mice were injected with Annexin Vivo 750 (100 µL i.v.) (Perkin Elmer) 1.5h before harvesting. After i.v.injection of Annexin Vivo 750 (Perkin Elmer), HT1080 H2B-mCherry tumors were harvested and embedded in optimal cutting temperature (O.C.T.) compound (Sakura Finetek). The serial frozen sections (thickness: 6 µm) were prepared and terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining (DeadEnd Fluorometric TUNEL System, Promega) was performed according to the manufacturer's protocol. The slides were coverslipped using a mounting medium with DAPI (Vector Laboratories, Inc.). The adjacent sections were imaged using a Nikon 80i microscope (Nikon Incorporated). Images from adjacent sections were overlayed using Fiji/ImageJ 1.45r (National Institutes of Health).

### *Image processing*

Microscopy images were processed using a collaborative digital filter using alpha root processing, to reduce noise and at the same time enhance image details for subsequent visual analysis. The algorithm is described in detail in [1]. Noise removal was achieved by a three step algorithm:1) Anscombe transformation; 2) collaborative filtering for Gaussian noise; 3) inverse Anscombe

transformation. Step 1: In order to remove the signal dependent component of the noise a variance-stabilizing transform was applied to the images. Anscombe variance stabilization transform aims at transforming the recorded signal which is Poissonian distributed to a noise made proximal to a normal distribution with unitary variance. Anscombe transform is defined as:

$y = 2\sqrt{x + \frac{3}{8}}$ , where x is the signal recorded by the microscope CCD detectors while y is the Anscombe transformed signal. Step 2: Gaussian noise can be considered additive, therefore each noisy image $I_{noisy}(x, y)$ can be expressed as follows: $I_{noisy}(x, y) = I(x, y) + n(x, y)$ , where $I(x, y)$ is the noise free image and $n(x, y)$ the additive corruptive Gaussian noise; $x, y$ are the coordinates of the pixels position within the image. The advantage of exploiting the Anscombe transform relies on the possibility to use a number of de-noise filters designed on such a distribution. Among the available filters we used one that exploits an important property that biomedical images exhibit, the auto-similarity. Such a state of the art denoise filter named BM3D has also been proven to be effective in critical conditions like in Optical Projection Tomography[1], where the amplification of the noise due to the Filter Back Projection reconstruction algorithm is an issue[2]. BM3D is based on the assumption that fragments of a noise-free image are similar, therefore a spectrum of packed similar fragments can be better approximated by fewer spectrum elements rather than a single image fragment. Operatively, each image is split into overlapping patches called reference blocks. For each reference block a block-matching algorithm was used to find similar patches within a 39x39 window, centered in the middle of the reference block. A stack of patches was then formed with the most similar closer to the reference one. This guarantees for a stack of patches' spectrum to be distributed over a few coefficients (with high values due to the total amount of signal energy), leading to a so called sparse representation. Contrariwise noise cannot be expressed with a few coefficients due to its characteristics to be uncorrelated with itself. This process allows a very high sparse representation of the stacks. Therefore since similar stacked image patches are compressible, i.e. they can be transformed into a domain where they are sparse, and since white noise is not, a hard threshold is effective in such a domain to reduce

the noise. Therefore, each 3D stack was transformed back along the packing direction to get the spectrum of the de-noised patches. De-noised image fragments were then processed by Alpha rooting technique. Alpha rooting is commonly applied to orthogonal transformation as in our case and works by modifying the spectra coefficient in order to increase high frequencies of the power spectrum. The technique is effective since image details are related to high frequency power spectrum coefficients. Such coefficients are obtained computing the alpha root of the absolute value of ratio coefficient/DC component. Afterwards, the result is rescaled by the magnitude DC component multiplied by the sign of the processed coefficient. If the DC component was equal to zero no alpha rooting was applied to that image fragment spectrum. Alpha rooting usually suffers from noise amplification, a drawback here avoided thanks to the thresholding used for denoising. Herein alpha varied from 1.2 to 1.3 when image crisping was applied. Finally, the 2D spectra were transformed back and the resulted de-noised patches weighted and fused together. Step 3: Filtered images were next transformed by the exact unbiased inverse transform to achieve optimal results in the whole image dynamic range including the case of low signal conditions. The implemented filter permitted very efficient noise removal while at the same time preserving and enhancing image features (e.g. edges).

## Supplementary Figure Legends

**Fig. S1: Colocalization of IMS-RP with full-length Smac/DIABLO and AIF. A.** MDA-MB-231 IMS-GFP cells were fixed with paraformaldehyde and stained with antibodies against GFP and Smac/DIABLO, to show colocalization of both truncated and full length Smac within the mitochondrial IMS. **B.** MDA-MB-231 IMS-GFP cells were fixed briefly with cold acetone (to preserve the integrity of the GFP) and stained with antibodies against apoptosis inducing factor (AIF), a protein that also localizes to the mitochondrial IMS. Scale bars are 25 μm.

**Fig. S2: Full-length movies of montaged images (shown in Figs. 1 and 2).** Images of MDA-MB-231 cells treated with ABT-263 (A) or PANC-1 cells treated with TRAIL (B) in culture were acquired each minute for approximately 5 hours. MOMP occurs between 5-7 seconds in the MDA-MB-231 cell (A), and 22-23 seconds in the PANC-1 cell (B). The cells of interest are designated with red asterisks.

**Fig. S3: Histological analysis of HT1080 H2B-mCherry tumors stained with Annexin V.** Images from one 6 μm-thick tissue section of HT1080 H2B-mCherry tumor (red nuclei), fixed and stained with DAPI (blue) and TUNEL (green) were merged with images from the same field of view from an adjacent 6 μm-thick tissue section that was left unfixed, in order to preserve the integrity of Annexin Vivo 750 signal (magenta), which is eliminated upon fixation. Annexin Vivo 750 appears at the tumor periphery, whereas TUNEL staining occurs throughout the tumor (bottom right image). Images were acquired at 20x magnification.