

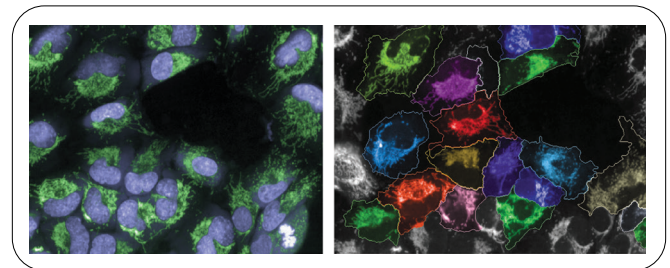
Phenotypic characterization of mitochondria in breast cancer cells using morphology and texture properties

High content image analysis

Mitochondria play a variety of important roles in eukaryotic cell physiology. In addition to producing ATP, they also regulate various cellular processes such as substrate utilization, redox homeostasis, intracellular calcium regulation and biosynthesis of macromolecules, as well as critical non-metabolic processes, particularly apoptosis. Mitochondria are known to undergo dramatic changes in morphology as cells grow, divide, and die. Mutations that affect mitochondrial morphology can affect metabolic functions as well as the cell's sensitivity to apoptotic stimuli.¹ However, the relationship between mitochondrial structure and function remains unclear.

It has long been thought that mitochondrial defects play an important role in the development and progression of cancer, and many of the cell growth and survival pathways targeted by anti-cancer drugs are mediated through mitochondria. However, it is not known how changes in mitochondrial shape and organization can lead to cancer, or how the altered form of cancer cell mitochondria can be exploited for therapeutic benefit.

In this case study, Dr. Chris Bakal and Dr. Julia Sero from the Institute of Cancer Research (ICR) in London, have validated the use of advanced morphological and texture analysis features in the Columbus™ image data storage and



analysis system* to quantify the effects of chemotherapeutic compounds on mitochondria morphology in breast cancer cells.

As well as advancing the understanding of mitochondrial biology in cancer, this research may contribute to the design of new strategies for effective cancer prevention, diagnosis, and treatment.

Quantification of mitochondrial structure

Mitochondria are complex networks that are highly variable, both between cell lines and within cell populations. Reliably quantifying the structures of these organelles therefore represents a considerable analytical challenge, requiring both excellent image resolution and the ability to extract hundreds of different parameters that can accurately describe complex forms.

In this study, images were acquired using the Opera® high content screening system and imported into the Columbus system for analysis of mitochondrial structures.

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*Columbus Image Data Storage and Analysis System has now been superseded by Signals Image Artist™ image analysis and management platform.

The Columbus system allowed researchers to measure hundreds of morphological and texture features for thousands of individual breast cancer cells in different treatment conditions. This led to the detection of changes and trends in diverse mitochondrial populations that could not be discerned by eye.



"I had used the Opera system in a previous laboratory, and wanted to bring the same technology to my current research because I believe it is really the only microscope on the market that is capable of generating high quality images in high throughput".

- Dr. Chris Bakal

STAR morphology and SER texture features

In this study, in addition to morphological features such as cell area, roundness, length, width and width/length ratio, STAR morphology and SER texture features were also measured for each cell (Figure 1). SER texture features measure local patterns of pixel intensity, which can reveal the structure of mitochondria even in high-signal areas around the nuclei (Figure 1 C-D), and can distinguish between, for example, punctate and tubular networks. STAR morphology is a set of algorithms which offer a statistically robust set of properties for classifying phenotypes by comprehensively describing cell morphology and the distribution of intensity within regions. The STAR method includes the ability to calculate **S**ymmetry properties, **T**hreshold compactness, **A**xial properties, **R**adial properties, and profile.

Advantages of STAR and SER features

The advantage of using STAR features in this analysis is that they gave important information about subcellular localization and distribution of mitochondria within cells. Cell profiles are useful for determining whether mitochondria are localized to the juxtamembrane (Figure 1E), cytosolic (Figure 1F), perinuclear, outer nuclear and/or

inner nuclear zones. Symmetry features can detect whether mitochondria are polarized or more evenly distributed. Radial features measure how close the mitochondria are localized to the cellular mass center and how homogenous their distribution is towards the cell border. Compactness features provide a measure of how tightly packed or "lacy" the networks are. STAR features were calculated for the MitoTracker® channel and for each of the SER-filtered images. Together, these features provide a highly detailed quantitative description of highly complex and variable cellular structures.

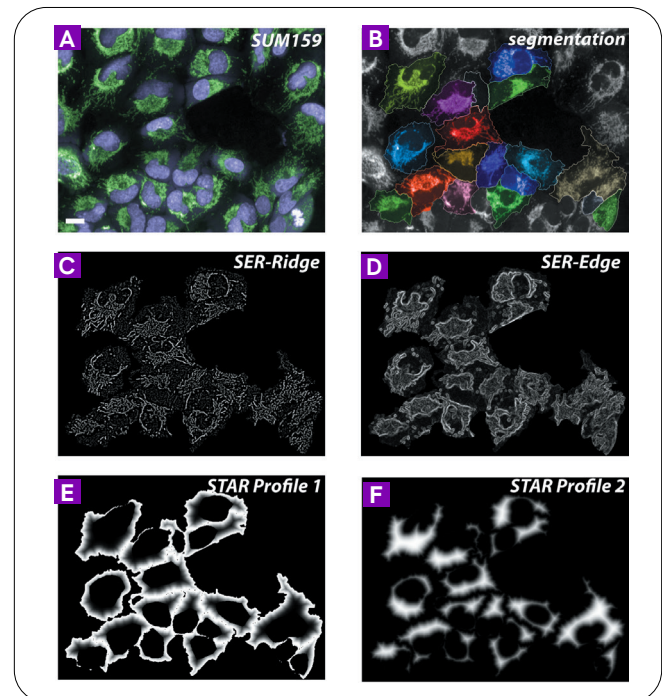


Figure 1. Image analysis using SER texture and STAR morphology features in the Columbus system. A) Raw image of SUM159 breast tumour cells. Nuclei are labelled with Hoechst (purple) and mitochondria with MitoTracker® (green); scale bar, 10 µm. B) Segmentation of cell bodies using the Columbus system. C-D) Output images following application of two SER texture features ('SER-Ridge' and 'SER-Edge') to the cells highlighted in (B). E-F) STAR profiles that define juxtamembrane (E) and cytosolic (F) regions of the cells highlighted in (B).

Experimental background

SUM159 breast tumour cells were cultured in 384-well plates. After 48 hr, the mitochondrial label MitoTracker® (Invitrogen) was added for 15 min, then removed and replaced with fresh media. Different drug treatments* (rotenone, mifepristone dexamethasone and two concentrations (10 nM and 50 nM) of tamoxifen) were then added to replicate wells for 24 hr. Following drug treatment, cells were incubated with a vital dye to label the cytoplasm

and then fixed with warm paraformaldehyde, prior to imaging on the Opera system. Images of control cells (no drug treatment), illustrating the phenotypic diversity of

mitochondria within the cell line, are shown in Figure 2A. Representative images from each drug treatment group are shown in Figure 2B.

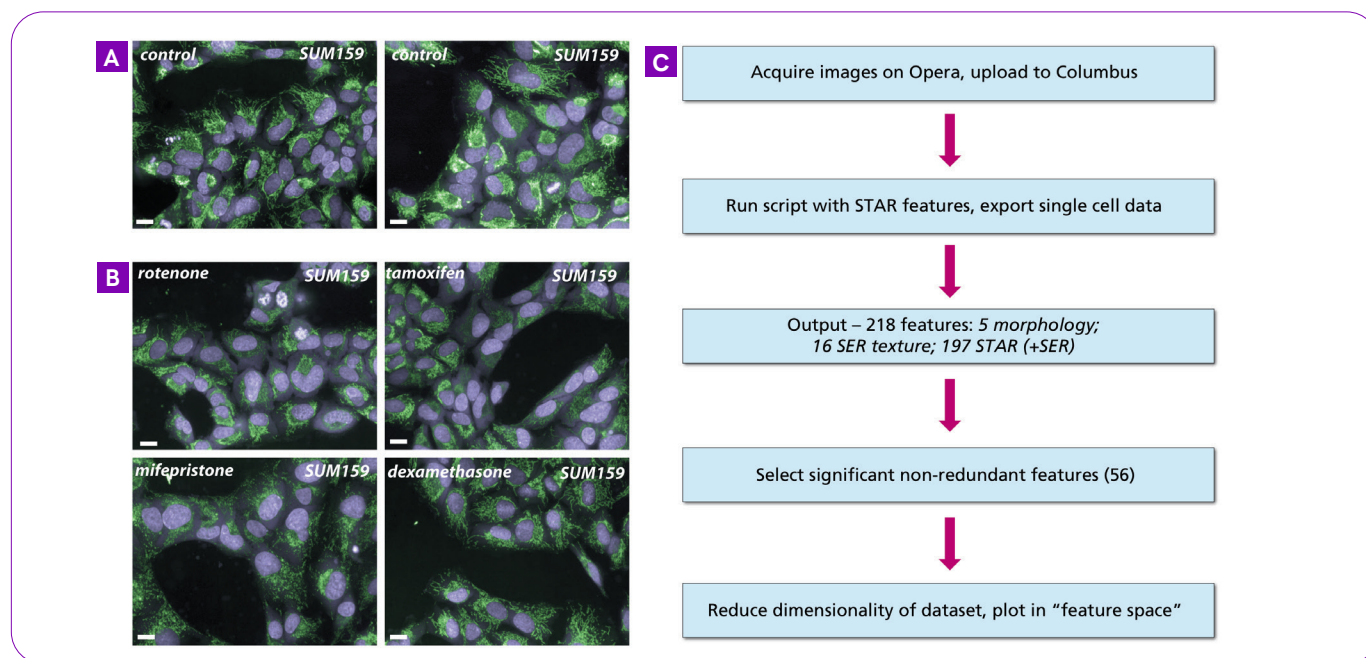


Figure 2. Profiling the mitochondrial morphology of SUM159 breast tumour cells with and without drug treatments. A) Images of control cells (no drug treatment), illustrating the range of morphologies present in the untreated population. B) Cells treated with rotenone, tamoxifen, mifepristone or dexamethasone. Mitochondria are labelled with MitoTracker® (green) and nuclei are stained with Hoechst (purple); scale bar, 10 μ m. C) Workflow for data acquisition and analysis.

Images were then imported into the Columbus system for analysis. Following image segmentation to identify the nuclei and cell bodies, morphology, STAR and SER texture analysis features were calculated. From a total of 7275 single cells, 5 cell morphological features, 16 SER texture features (on the cytoplasm and whole cell regions), 21 STAR features and 176 STAR+SER features were extracted, and single cell data exported.

Extracting meaning from the data

From this extensive list of STAR+SER features, a subset of non-redundant features was selected for further analysis using hierarchical clustering to identify correlated features. For each set of features with a correlation coefficient of > 0.7 , the feature with the lowest median p -value across each pair of treatment conditions was selected. This process yielded a subset of 56 significant features, of which 3 were morphological, 2 were cytoplasm SER, 12 were STAR and 39 were STAR-SER (detailed in Figure 3A). The mean values across four features are shown in Figure 3B. The mifepristone treatment group was significantly

different to the control group in 48 features, followed by rotenone (43), dexamethasone (17) and finally tamoxifen (12 for the 50 nM group and 11 for the 10 nM group). Only two STAR-SER features, 'Cell Symmetry 02-SER-Valley' (Figure 3B, grey asterisk) and 'SER-Dark', varied significantly between the two doses of tamoxifen. However, no single feature was sufficient to discriminate between all treatment conditions.

To reduce the dimensionality of the data set and consider all 56 features together, a Principle Component Analysis (PCA) was performed after normalizing all feature values to Z-scores. PCA transforms the data points (features) for each object (cell) into a new set of coordinates where the first principle component (PC1) accounts for the most variance, the second principle component (PC2) accounts for the second most variance, and so on. Using PCA, the distance of each treatment condition from the control in "feature space" was calculated. Figure 3C shows the Euclidean distances of PC coordinates from the control using morphological features only, or coordinates from the 56 selected STAR+SER features.

Although Euclidean distances illustrate the degree of difference between populations, they do not reveal the nature of the changes. For example, cell morphology was affected to a similar degree by rotenone and mifepristone but the former caused cells to become slightly smaller and rounder, whereas the latter induced spreading and elongation. Plotting the mean PC1 (x) and PC2 (y) coordinates for each treatment condition shows the relative positions of each treatment in "feature space", which indicates whether

treatments had similar or different effects on morphology (Figure 3D). The PCA coordinate plot shows that mifepristone and rotenone had opposite effects on mitochondrial morphology. Furthermore, while dexamethasone had a less dramatic effect than mifepristone, the two treatments are located in the same quadrant of "feature space", indicating similar morphological changes. This is consistent with the fact that both drugs act on the same receptor, albeit with different affinities and modes of action.



Figure 3. Analysis of mitochondrial morphology using STAR and SER features. A) 56 non-redundant features were identified for use in cell classification and are shown grouped by type. B) Mean values (\pm SEM) of each treatment condition for four selected features ($n = \sim 1100$ cells per condition). Different features varied significantly ($p < 0.01$) between control and drug treated populations (black asterisks). One of these ('Cell Symmetry 02 SER-Valley') was significantly different between populations treated with different doses of tamoxifen (grey asterisk). C) After performing a PCA on cell morphology features alone (blue) and the 56 selected STAR+SER features (red), Euclidean distances from the control in "feature space" were calculated for the mean coordinates of each treatment condition. The STAR features calculated on the texture-rendered images provided much higher Euclidean distances and were more effective at distinguishing between cells in different treatment conditions. D) Plot of the mean first (x axis) and second (y axis) PC for each treatment condition relative to control (0,0) in "feature space".

Conclusion

This case study validates the use of STAR morphology and SER texture features to quantifiably characterize mitochondrial morphology and to detect changes induced by drug treatments. In spite of the high degree of phenotypic variability within cell populations, researchers were able to distinguish not only the effects of different compounds on mitochondrial organization, but also the effects of two doses of the same drug using this method.

While the effect of one drug (mifepristone) on mitochondrial morphology is clear from images (Figure 2 A-B), the effects of the other treatments, though statistically significant, are more subtle and would be difficult or even impossible for human investigators to detect. Conversely, in such diverse populations there is a danger of extrapolating phenotypic effects based on a few unusual cells (the von Restorff effect). Detecting deviations from the normal range of mitochondrial morphology therefore requires the measurement of hundreds of single cells across many variables, which is only feasible using high content automated image analysis. These methods will also allow investigators to analyze the degree of heterogeneity within treatment groups, to detect subpopulations, and to measure the percentage of cells that are affected by treatment (phenotypic penetrance).

This study illustrates the power of high content automated image analysis, using the Opera system and the texture and STAR features of the Columbus system, to quantify complex organelle morphologies. Importantly, data of this type can be gathered in large-scale formats, suggesting potential applications in drug development and genetic screens.

*Drug treatments

Rotenone: an inhibitor of mitochondrial Complex I (NADH dehydrogenase), which induces mitochondrial dysfunction and superoxide free radical production.

Mifepristone (or RU-486): a glucocorticoid receptor (GR) antagonist/partial agonist.

Dexamethasone: a potent GR activator. Steroid hormone receptors such as GR can regulate mitochondrial function directly, by regulating the transcription of mitochondrial genes, and through interactions with other mitochondria-localized proteins, such as the anti-apoptotic protein Bcl-2.^{2,3,4}

Tamoxifen: an estrogen receptor (ER) inhibitor widely used to treat ER α ⁺ breast cancer, but 5-10% of ER α ⁻ tumours also respond to tamoxifen therapy. This is likely due to the expression of ER β ,⁵ which has been shown to localize to mitochondria in several cell types.^{6,7}

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Acknowledgments

Many thanks to Dr. Julia Sero for authoring this manuscript. Thanks also to Dr. Chris Bakal, Dr. Karin Boettcher (Revvity) and Dr. Sarah Piper (Revvity) for comments on the manuscript. This work was supported by the Wellcome Trust, BBSRC, Cancer Research U.K. and Revvity, Inc.

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