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# Kinetic measurements of intracellular ATP levels in co-culture models using live-cell analysis

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## Summary & Impact


- Differential metabolic requirements of tumor cells have gained recognition as attractive therapeutic targets (e.g. glutaminase1 (GLS1) inhibitor CB-839 in clinical trials for a variety of indications, including triple-negative breast cancer (TNBC)).
- Standard approaches to monitoring drug induced metabolic perturbations are limited to endpoint assays that lack cell-specific data in complex co-culture models and provide limited kinetic information.
- The Incucyte® ATP Assay provides a live-cell approach to image and analyze cytosolic ATP levels of tumor cells in mono- or co-culture with stromal cells over time.
- Across all TNBC lines tested, CB-839 treatment resulted in a reduction in ATP that was sustained for the duration of the 3-day time course. In contrast, most receptor-positive cell lines displayed little to no effect of GLS1 inhibition.
- Co-culture with CCD-1086SK fibroblasts attenuated the effect of CB-839 in TNBC cell lines, an effect which was not due to drug buffering.
- Visualization tools included in the Incucyte® ATP Analysis Software Module provide a quick, qualitative assessment of data across the assay plate and a view into the heterogeneity of treatment responses.
- Measurements of proliferation, cell death, and mitochondrial membrane potential enabled by the Incucyte® Metabolism Optical Module and associated reagents can provide additional insight and supporting data.


## Incucyte® System for continuous live-cell analysis: Methodology


**Incucyte® Metabolism Optical Module**
A single, flexible platform designed to combine the analysis of metabolism with additional phenotypic assays
– End-to-end solution: reagents, protocols, plus built-in acquisition and analysis software modules.
– Uninterrupted incubation to reduce biological artifacts.

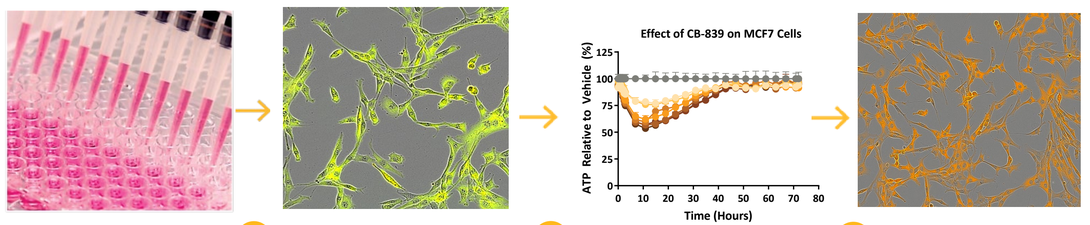
NEW: Metabolism Optical Module

**IncuCyte® ATP Software Module**
Fast, flexible and powerful control hub for continuous live-cell analysis comprising image acquisition, processing and data visualization.
– Color scaled visualization of cytosolic ATP levels
– Graphing of normalized kinetic data

NEW: Incucyte® ATP Analysis Software Module

**Incucyte® Metabolism Reagents**
A suite of non-perturbing cell labelling and reporter reagents.
– CytoATP, CytoATP Non-binding Control, and NucLight Orange Reagents
– Mitochondrial Membrane Potential (MMP) Kit
– Annexin V and Caspase 3/7 Cell Health Reagents

NEW: Incucyte® CytoATP and CytoATP Non-binding Control Lentivirus Reagents, Incucyte® MMP Orange Kit



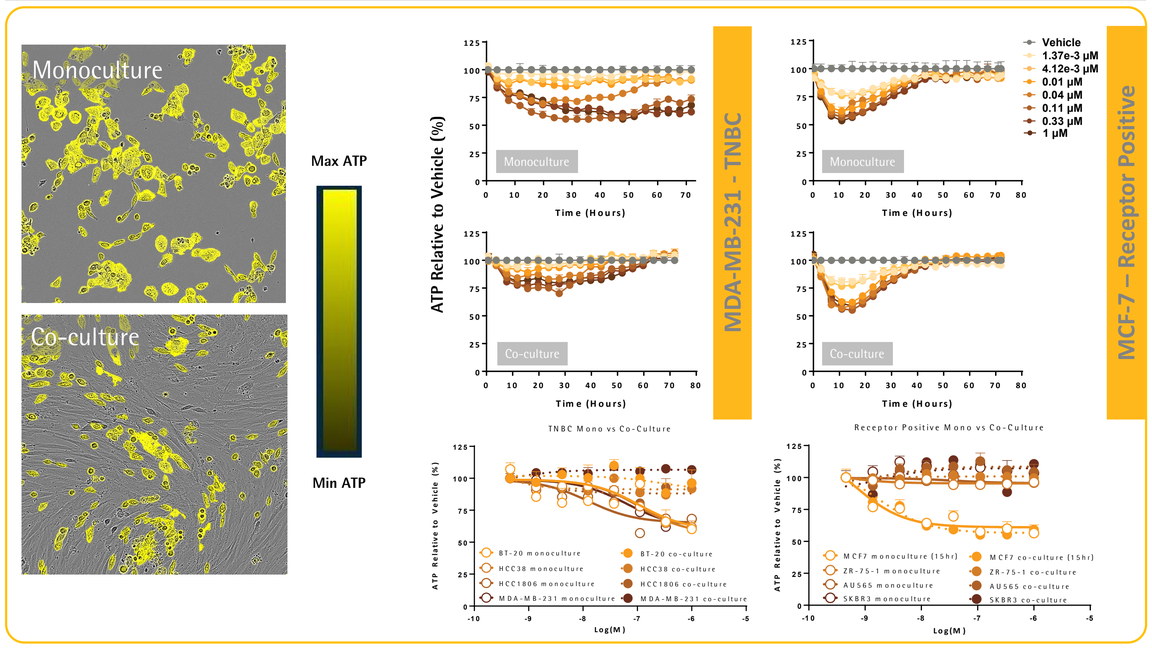

1 **Prepare assay plate**
Infect cells with Incucyte® CytoATP Reagents.

2 **Acquire images**
Capture images over time in a physiologically relevant environment.

3 **Analyze metabolic changes**
Quantify metabolic changes in the same population of cells with fully integrated kinetic analysis.

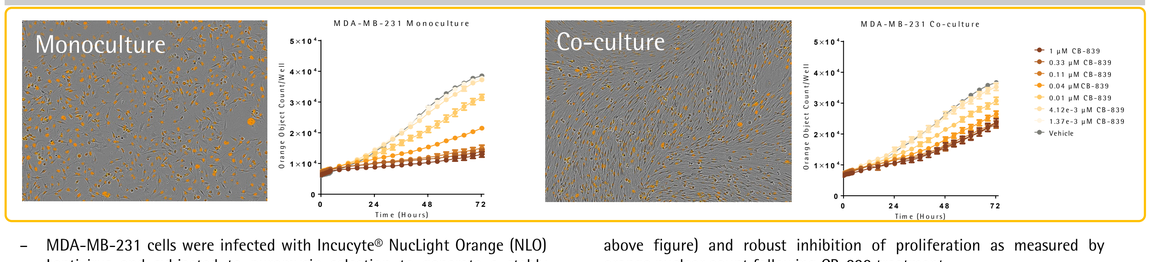
4 **Confirm cell morphology**
Associate changes in cell morphology with metabolic readouts.

## Cell type-specific ATP measurements in co-culture



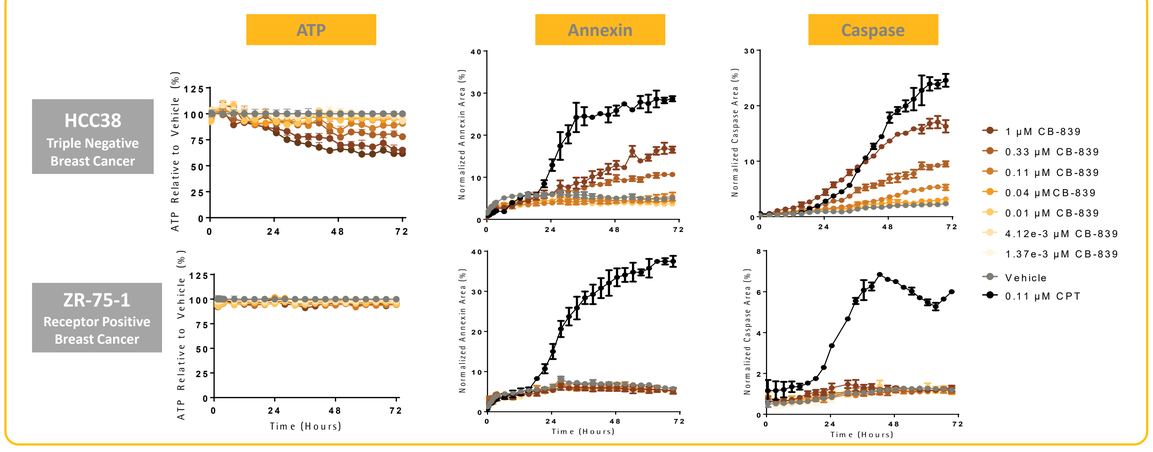

- TNBC or receptor-positive breast cancer cell lines stably expressing CytoATP or Non-binding Control indicators were seeded in the presence and absence of CCD14086SK fibroblasts (8K cells/well) and treated with CB-839 (1.37 nM-1 µM).
- Identification of cellular changes in ATP (color scaled masking) is performed using the integrated Incucyte® ATP Analysis Software Module.
- Masked images provide visualization of lower ATP in HCC1806 cells treated with 330 nM CB-839 in monoculture compared to those in co-culture.
- Kinetic data shows a sustained depletion of CytoATP in TNBC lines grown in monoculture following CB-839 treatment, whereas receptor-positive cell lines displayed little to no effect or, in the case of MCF7 cells, a transient decrease followed by full recovery.
- Co-culture with stromal cells mediated resistance to CB-839 in a panel of TNBC cells, while effects on receptor-positive cell lines were unaltered between the two conditions (72 h time points shown except where indicated).
- This effect was not due to drug buffering, as the receptor-positive cell line MCF7 showed overlapping concentration-response curves in both conditions (15 h time point shown to capture transient ATP depletion).

## Cell type-specific measurement of proliferation in co-culture



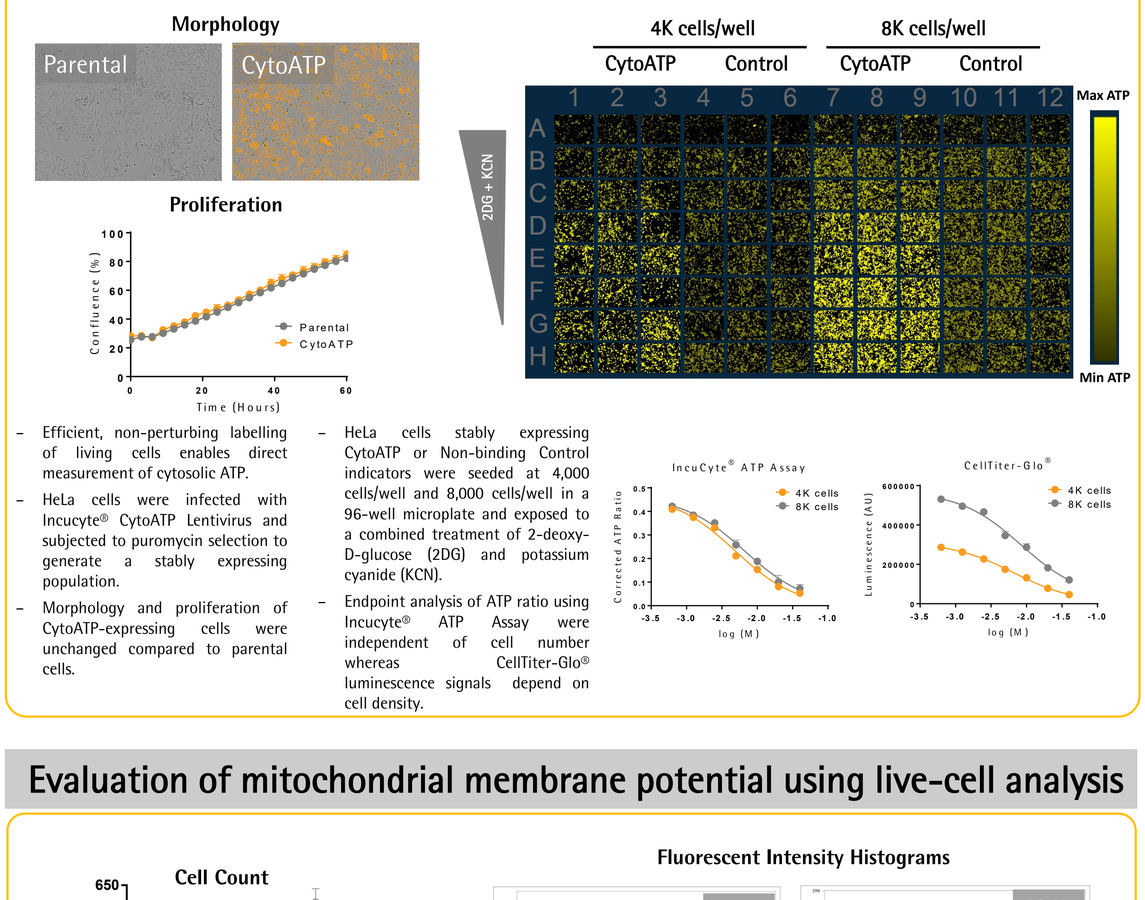

- MDA-MB-231 cells were infected with Incucyte® NucLight Orange (NLO) Lentivirus and subjected to puromycin selection to generate a stably expressing population.
- MDA-MB-231 (TNBC) cell line displayed a sustained depletion of ATP (see above figure) and robust inhibition of proliferation as measured by orange nuclear count following CB-839 treatment.
- Co-culture of MDA-MB-231 cells with CCD14086SK fibroblasts mitigated ATP depletion and reduced the anti-proliferative effect of CB-839.

## Measure apoptosis with Annexin V and Caspase 3/7 Orange Reagents



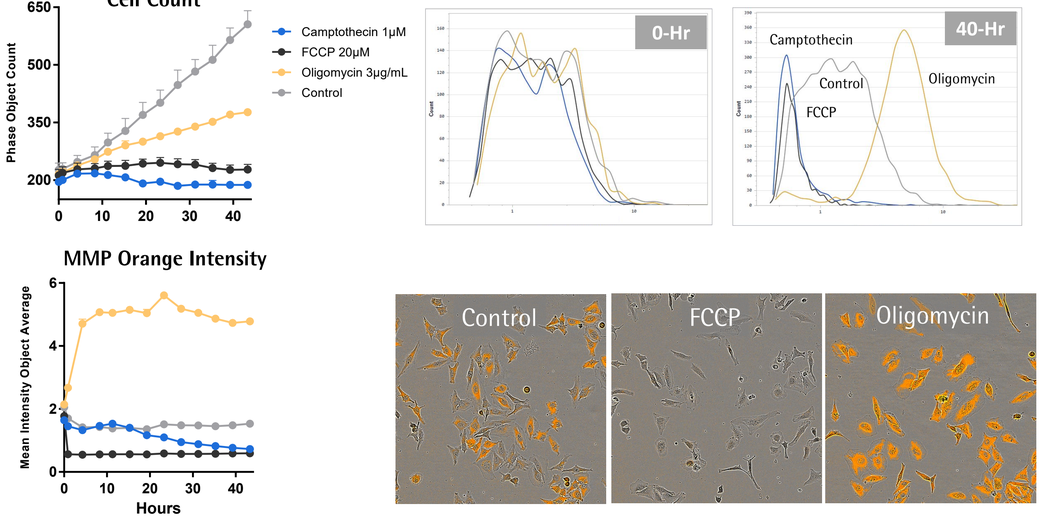

- TNBC (HCC38) and receptor-positive (ZR-75-1) breast cancer cell lines stably expressing CytoATP or Non-binding Control indicators show expected ATP response when treated with CB-839 (1.37 nM-1 µM). Specifically, CB-839 selectively depletes ATP in HCC38 cells (TNBC).
- IncuCyte® Annexin V Orange and IncuCyte® Caspase-3/7 Orange reagents are non-perturbing, mix and read apoptosis reagents that provide supporting cell health data.
- Apoptosis was observed over time selectively in the TNBC cell line treated with CB-839, while only the positive control (Camptothecin, CPT) generated a response in the receptor-positive cell line.

## Automated measurement of ATP independent of cell number




- Efficient, non-perturbing labelling of living cells enables direct measurement of cytosolic ATP.
- HeLa cells were infected with Incucyte® CytoATP Lentivirus and subjected to puromycin selection to generate a stably expressing population.
- Morphology and proliferation of CytoATP-expressing cells were unchanged compared to parental cells.
- HeLa cells stably expressing CytoATP or Non-binding Control indicators were seeded at 4,000 cells/well and 8,000 cells/well in a 96-well microplate and exposed to a combined treatment of 2-deoxy-D-glucose (2DG) and potassium cyanide (KCN).
- Endpoint analysis of ATP ratio using Incucyte® ATP Assay were independent of cell number whereas CellTiter-Glo® luminescence signals depend on cell density.

## Evaluation of mitochondrial membrane potential using live-cell analysis




- HeLa cells were treated with the mix-and-read Incucyte® MMP Orange Reagent to detect live-cell changes in mitochondrial membrane potential.
- Histograms show mean fluorescent intensity for control and drug-treated wells at 0 and 40hrs. Oligomycin increases fluorescent intensity (i.e. MMP) while FCCP and Camptothecin show a reduction.
- Proliferation is also measured in parallel by using the Cell-by-Cell Analysis software (i.e. Phase Object Count).
- Images show the MMP Orange Reagent used with control compounds, Oligomycin and FCCP.

## Acknowledgments

We thank Hinnah Campwala for providing the NLO figures and John Rauch for generating TMRM data.