APPLICATION NOTE

Validation of PROTEOSTAT® Aggresome Detection Kit and ROS-ID® Total ROS Detection Kit via the IncuCyte S3 and Operetta CLS Automated Imaging Platforms.

Svetlana N. Kononenko, Ph.D. | Aleksander D. Szymaniak, Ph.D. | Aaron J. Wolfe, Ph.D. | Ichor Life Sciences
Anthony Romeo, Ph.D. | Application Scientist | Enzo Life Sciences

PROTEOSTAT® Aggresome Detection Kit (ENZ-51035)
ROS-ID® Total ROS Detection Kit (ENZ-51011)

INTRODUCTION

The formation of protein aggregates and reactive oxygen species (ROS) within cells are two factors that play a critical role in cellular stress responses and the pathogenesis of various diseases, including neurological disorders (such as Parkinson's disease and Alzheimer's), cancers (such as solid tumors and leukemia), and cardiovascular pathologies. While aggresomes form when other proteasome systems are overwhelmed with misfolded or aggregated proteins, their formation is a double-edged sword. In a typical cell, aggresomes can serve as cytoprotective entities by means of sequestering misfolded structures in proteinaceous inclusions, aiding their further clearance by the autophagy-lysosome pathway. In the context of cancer, aggresome formation might facilitate cell survival under high metabolic activity. On the other hand, ROS are highly reactive molecules that are naturally produced during cellular metabolism, particularly in mitochondria. While ROS is essential for normal cellular signaling, excessive ROS production leads to oxidative stress, which can damage cellular structures, including proteins, lipids, and DNA. There is some overlap between aggresomes and ROS, as the formation of aggresomes is often triggered by oxidative stress, and ROS can be produced as a result of aggresome formation. These factors underscore the complexity of cellular stress management, and highlight the importance of studying these signals in research aimed at creating therapeutic strategies related to protein misfolding, neurodegeneration, and other cellular dysfunctions.

BENEFITS

- Validated cellular stress detection
- Precisely track cell responses to novel treatments
- Witness the exact moments cells react to stress, and what it means for future therapies

Enzo's PROTEOSTAT® Aggresome Detection Kit (ENZ-51035) and ROS-ID® Total ROS Detection Kit (ENZ-51011) are specifically designed to identify and visualize these phenomena in cells. The PROTEOSTAT® Aggresome Detection Reagent contained in the kit is a molecular rotor dye that specifically intercalates into the cross-beta spine of quaternary protein structures typically found in misfolded and aggregated proteins, which will inhibit the dye's rotation and lead to a strong fluorescence signal. This kit provides a rapid, specific, and quantitative approach to label aggresomes in cells, eliminating the need for artificial protein mutations. The ROS-ID® Total ROS Detection Kit directly monitors global levels of reactive oxygen species (ROS) in live cells. This kit includes the Oxidative Stress Detection Reagent (green) to monitor reactive oxygen and nitrogen species production in real time. This fluorogenic, cell-permeable detection probe reacts directly with a wide range of reactive species (hydrogen peroxide, peroxynitrite, and hydroxyl radicals), yielding a green fluorescent product.

Here, we demonstrate the application of Enzo's CELLESTIAL® fluorescence-based detection assays in two widely used carcinoma and glioblastoma cell lines, leveraging both live-cell and endpoint imaging techniques to capture complex cellular responses. The ROS-ID® Total ROS Detection Kit enables real-time monitoring of oxidative stress in glioblastoma cells using the IncuCyte® S3 live-cell analysis system (Sartorius). Similarly, the PROTEOSTAT® Aggresome Detection Kit is used to analyze aggresome formation in carcinoma and glioblastoma cells through high-content fluorescence imaging on the Operetta CLS™ automated platform (Revvity). These complementary approaches highlight the versatility of CELLESTIAL® kits and provide controls across different cell types, imaging platforms, and experimental workflows.

This work also features two cell lines with different morphologies that are commonly used in research, including live and endpoint image analysis, as well as normalization methods. Additionally, it highlights the exceptional performance of assay controls provided in each kit.

CELL LINES VALIDATED

U87MG (Human Glioblastoma)

A549 (Human Lung Carcinoma)

INSTRUMENTATION

Operetta CLS™:

The Operetta CLS™ (Revvity) is a state-of-the-art high-throughput microplate reader equipped with advanced spinning disk confocal imaging abilities. The automated objective platform enables seamless image acquisition of 2D cell cultures, 3D organoids, and entire tissue sections. The 8-LED emission sources and suite of water-immersion objectives yield high-resolution images. The hardware is coupled with Harmony software, which features advanced image analysis modules and proprietary machine learning technology. The features of the Operetta CLS™ allow it to tackle problems of high-content imaging with both 2D and 3D models.

IncuCyte® S3:

The IncuCyte S3 (Sartorius) is a live cell imaging cytometer featuring a library of validated reagents and data analysis modules, making it ideal for high-throughput screening. The versatility of the IncuCyte has been extensively verified in applications such as cell viability, chemotaxis migration, kinase activity, antibody internalization, immune cell infiltration, 3D organoid growth, and others. The robust hardware and intuitive user interface enable consistent data and reliable results.

MATERIALS AND METHODS

PROTEOSTAT® Aggresome Detection Kit (ENZ-51035)

A549 Cell Line:

A549, a human lung carcinoma cell line (Ichor Life Sciences cell culture bank), was maintained in Dulbecco's Modified Eagle Medium (DMEM) (Corning, 10-017-CV) supplemented with 10% Fetal Bovine Serum (FBS) (Corning, 35-015-CV) (complete medium). For the assay, cells were seeded in 96-well plate (Revvity, 6055302) (8,000 cells/well) and exposed to either 5 or 10 µM of MG-132 (Enzo-provided positive control for aggresome formation) for 18 hours. Cells were fixed in 4% PFA for 20 minutes at room temperature and permeabilized with 0.1% Triton in 1X PBS for 30 minutes, followed by several washing steps with 1X PBS (Corning, 21-040-CM). Cells were stained with a dual-stain cocktail containing an aggresome detection reagent and Hoechst for 30 minutes at room temperature, followed by several washes in 1X PBS. Plates were immediately imaged on the Operetta CLS high-content imaging system at 20X, air objective using Alexa 546 filter set (Ex. 530-560/ Em. 570-650). Quantitative evaluation of aggresomes was performed with Harmony 5.1 Spot Analysis.

U87MG Cell Line:

U87MG, a human lung carcinoma cell line (Ichor Life Sciences cell culture bank), was maintained in Dulbecco's Modified Eagle Medium (DMEM) (Corning, 10-017-CV) supplemented with 10% Fetal Bovine Serum (FBS) (Corning, 35-015-CV) (complete medium). For the assay, cells were seeded in 96-well plate (Revvity, 6055302) (8,000 cells/well) and exposed to either 5 or 10 µM of MG-132 (Enzo-provided positive control for aggresome formation), for 18 hours. Cells were fixed in 4% PFA for 20 minutes at room temperature and permeabilized with 0.1% Triton in 1X PBS for 30 minutes, followed by several washing steps with 1X PBS (Corning, 21-040-CM). Cells were then stained with the dual-stain cocktail containing aggresome detection reagent and Hoechst for 30 minutes at room temperature, followed by several washes in 1X PBS. Plates were immediately imaged on the Operetta CLS high-content imaging system at 20X, Air objective using Alexa 546 filter set (Ex. 530-560 nm/ Em. 570-650 nm). Quantitative evaluation of aggresomes was performed with Harmony 5.1 Spot Analysis.

Segmentation analysis with Harmony: (Analysis Sequence building blocks are denoted in Italic). The analysis followed a classical segmentation workflow, beginning with nuclei identification (Find Nuclei and Select Population to remove border objects) in the Hoechst channel, followed by cytoplasm segmentation (Find Cytoplasm) using the aggresome rotor dye (Alexa594 filter set). To enhance accuracy, background removal was performed using a sliding parabola filter (Filter Image), which isolated individual aggresomes and quantified their area (Find Spots), despite variability across cell types. A key strength of this assay setup is its ability to effectively segment cell populations based on morphological features while detecting aggresomes throughout the cytoplasm. Although aggresomes pre-

dominantly accumulate in the perinuclear region, they can also form in other areas of the cytoplasm and undergo microtubule-mediated transport. Thus, the selected carcinoma and glioblastoma cell lines provide a unique testing ground for segmentation algorithms due to their distinct morphologies—carcinoma cells exhibit a classical star-convex structure, while glioblastoma cells display an irregular shape—enhancing the robustness of fluorescence image analysis.

ROS-ID® Total ROS Detection Kit (ENZ-51011)

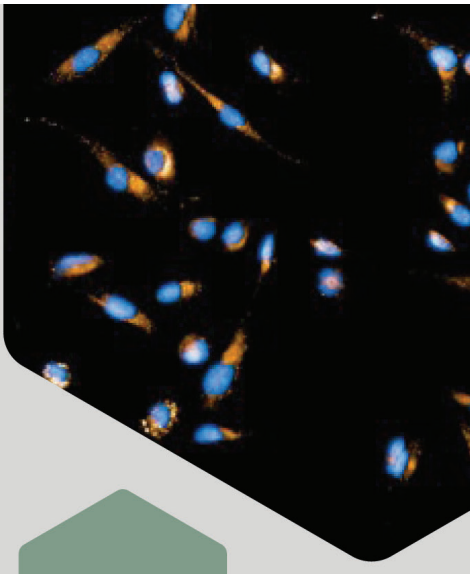
U87MG Cell Line:

Dulbecco's Modified Eagle Medium (DMEM) (Corning, 10-017-CV) supplemented with 10% Fetal Bovine Serum (FBS) (Corning, 35-015-CV) (complete medium). For the assay, cells were seeded in a 96-well plate (Revvity, 6055302) (12,000 cells/well) and allowed to attach overnight. Pyocyanin (Enzo-provided ROS inducer) was diluted to 2X (200-800 µM) and applied along with 2X of oxidative stress detection reagent (green) in IMDM (Gibco, 21056023) (phenol-free basal medium) supplemented with 1.5% FBS to monitor the production of total ROS. N-Acetyl-L-Cysteine (NAC, Enzo) was used at 10µM as a negative control for ROS production. Pyocyanin (Enzo-provided ROS inducer) was diluted to 2X (200-800 µM) and applied along with 2X of oxidative stress detection reagent (green) in IMDM (Gibco, 21056023) (phenol-free basal medium) supplemented with 1.5% FBS to monitor the production of total ROS. Live cells were then imaged every 20 minutes over a 2-hour period using the 488nm green channel and brightfield on the IncuCyte S3 live imaging system at a 10X objective. Data was analyzed with image segmentation using Sartorius software.

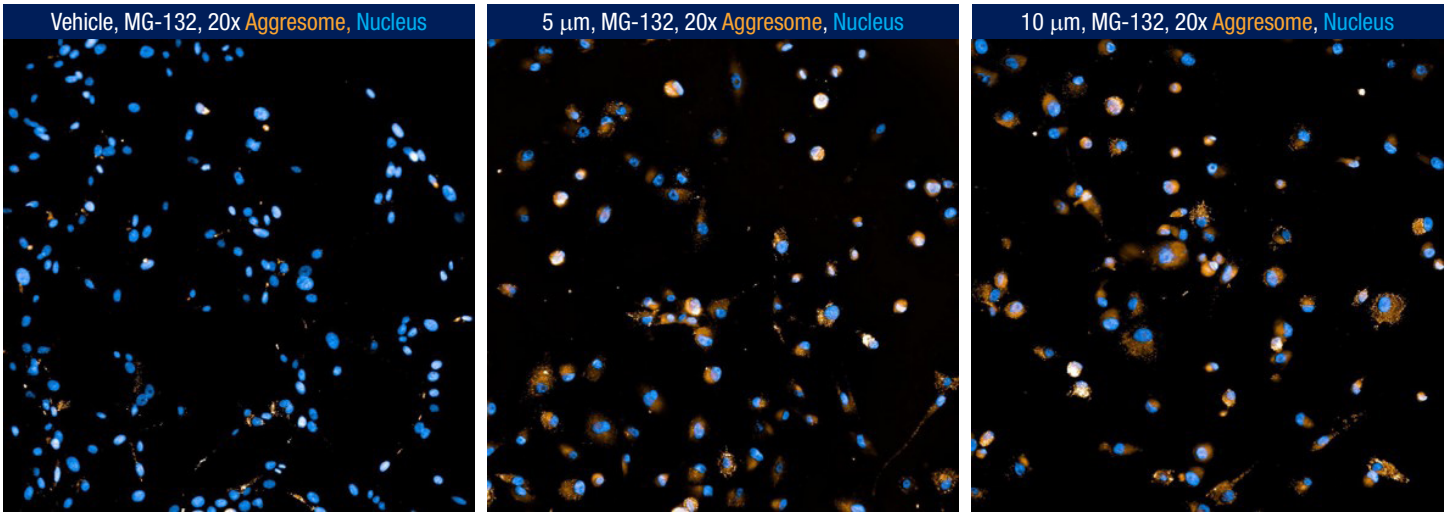
RESULTS

Aggresome formation is detected in U87MG and A549 cells after 18 hours of treatment with MG-132 (Figs. 1a, 2a). Quantification of images by both the number of aggresomes per nuclei and aggresome area per nuclei revealed significant differences in MG-132 treated cells when compared to untreated vehicle control cells (Figs. 1b-d, 2b-d).

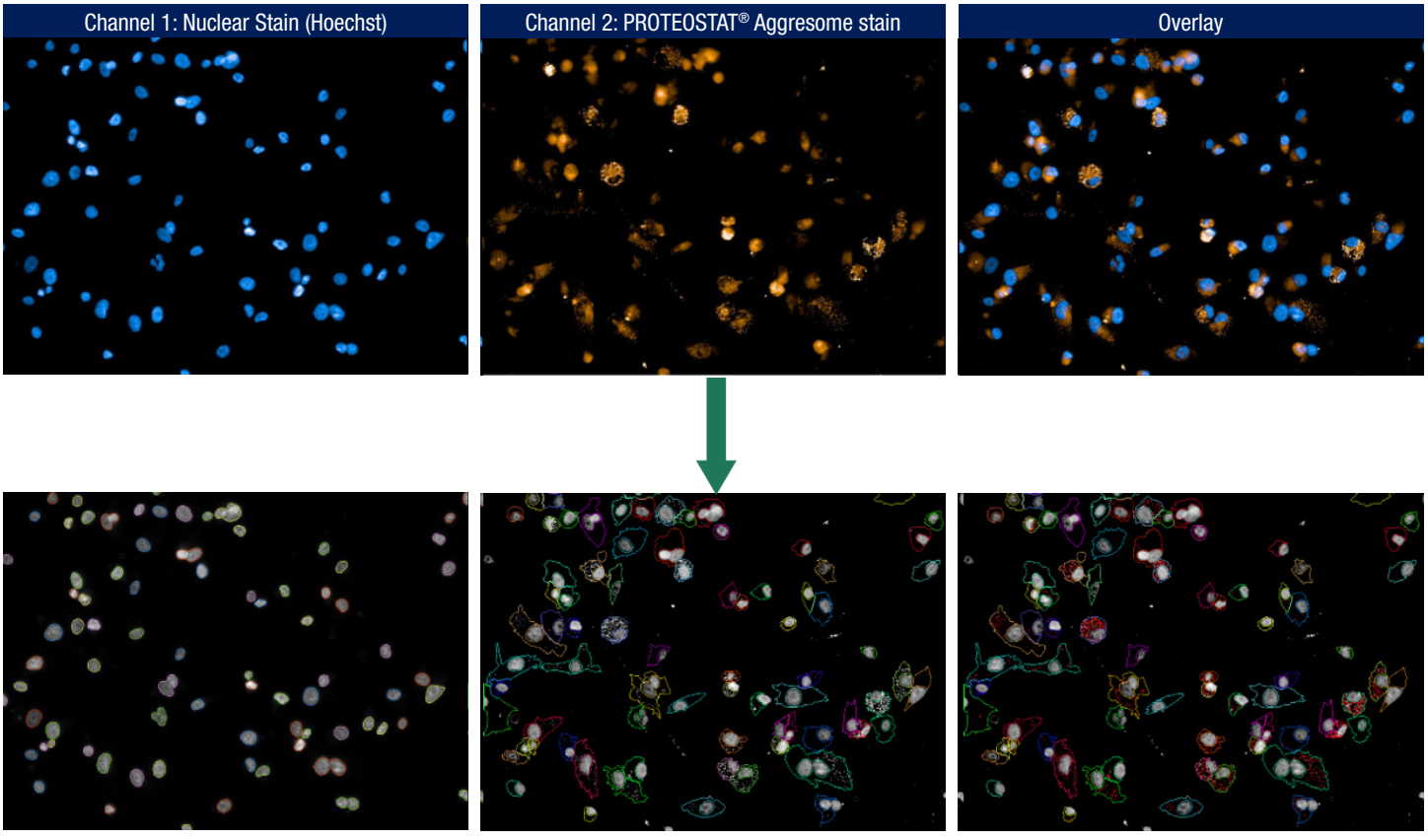
When treated with pyocyanin, U87MG cells show peak signals for ROS activity after about 40-60 minutes when compared to NAC control (Fig. 3a). This pattern holds true when calculating for both integrated intensity per well per green object (Figs. 3b and 3c), and integrated intensity area per well per phase area confluence (Fig. 3d). Treatment of U87MG cells with 1.0mM doxorubicin similarly led to detection of ROS after about 16 hours when compared to vehicle control (Figs. 4a and 4b). Dose amounts of 100nM and 500nM doxorubicin were not as effective at inducing ROS activity when monitoring integrated intensity per object count over time (Fig. 4b). This pattern holds true when calculating integrated intensity per confluence (Fig. 4c). There is also no significant change in confluency over time in the treated cells when compared to vehicle control (Fig. 4d).



1a.



1b.

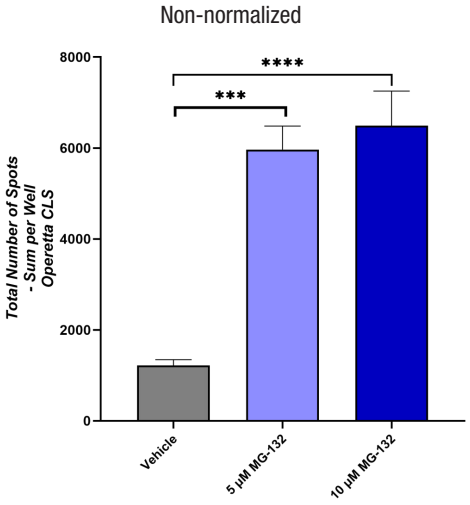


Segmented nuclei indicated with rainbow outlines around the nuclear stain. *Edge and incomplete objects are filtered out

Detected cytoplasm (based on channel 2) is outlined with rainbow borders and overlaid on "Sliding Parabola" filtered aggresome stain image

Detected Spots within the cytoplasmic outlines to indicates aggresomes (in red)

1c.



1d.

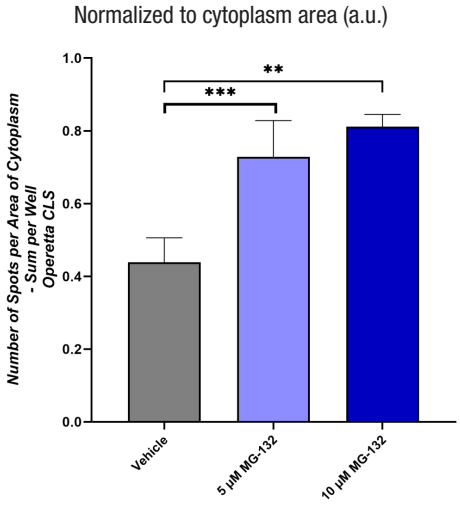
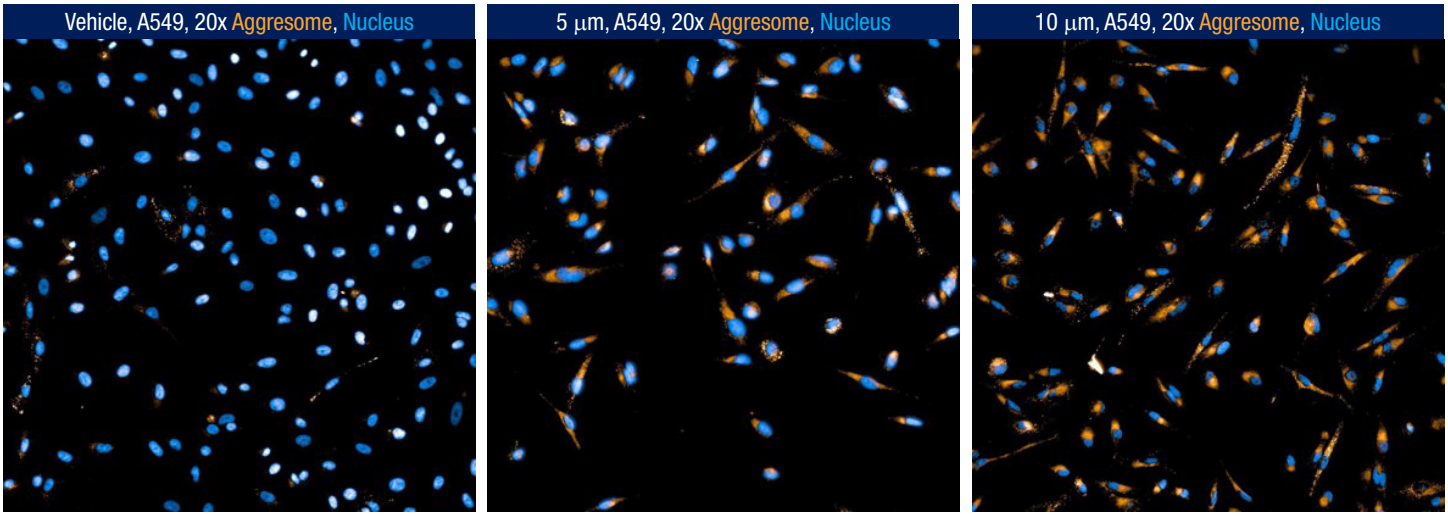
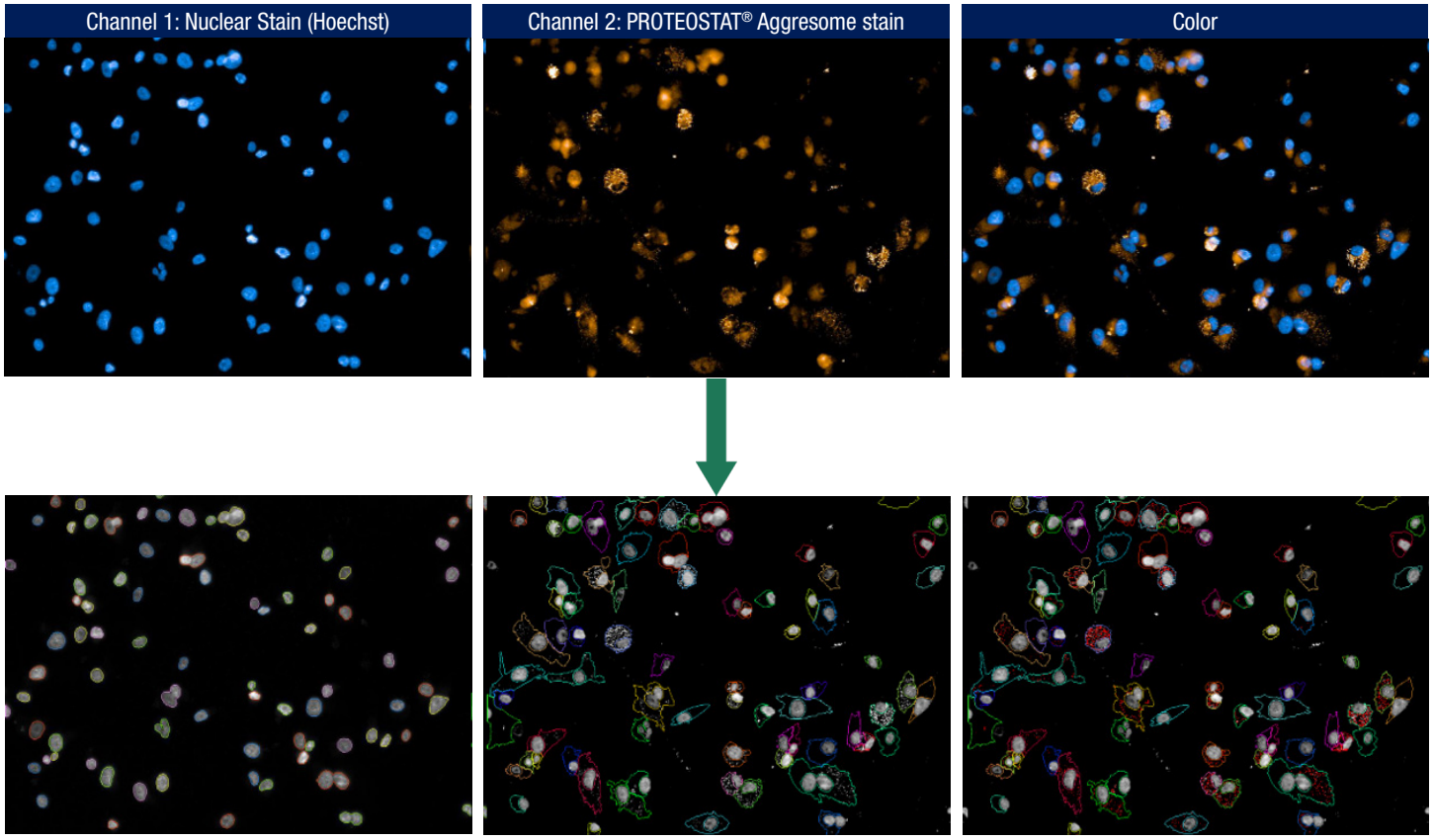


FIGURE 1: **Aggresome detection in U87MG cells.** a) Fluorescent images of vehicle vs 5µM and 10µM MG-132 treated (18 hours) U87MG cells. b) Representation of image segmentation performed by Operetta CLS™ to determine quantification in 1c and 1d. c) Quantification of images in 1a by number of aggresomes per nuclei detected in field of view. d) Quantification of images in 1a by total aggresome spot area per nuclei detected in field of view.

2a.



2b.

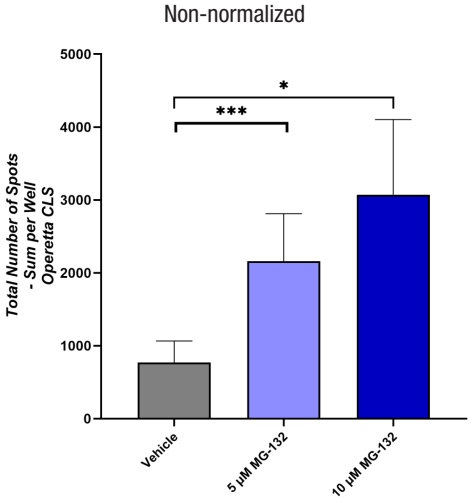


Segmented nuclei indicated with rainbow outlines around the nuclear stain. *Edge and incomplete objects are filtered out

Detected cytoplasm (based on channel 2) is outlined with rainbow borders and overlaid on "Sliding Parabola" filtered aggresome stain image

Detected Spots within the cytoplasmic outlines to indicates aggresomes (in red)

2c.



2d.

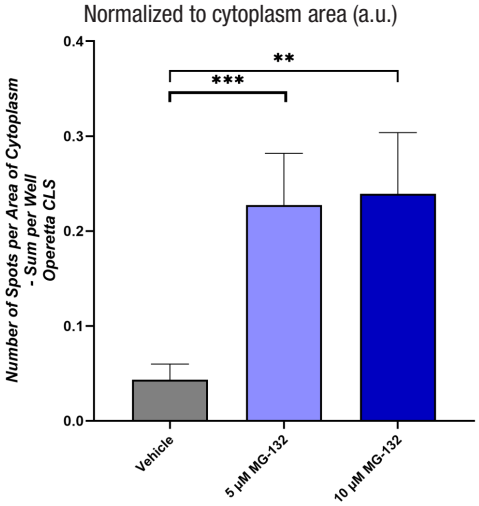
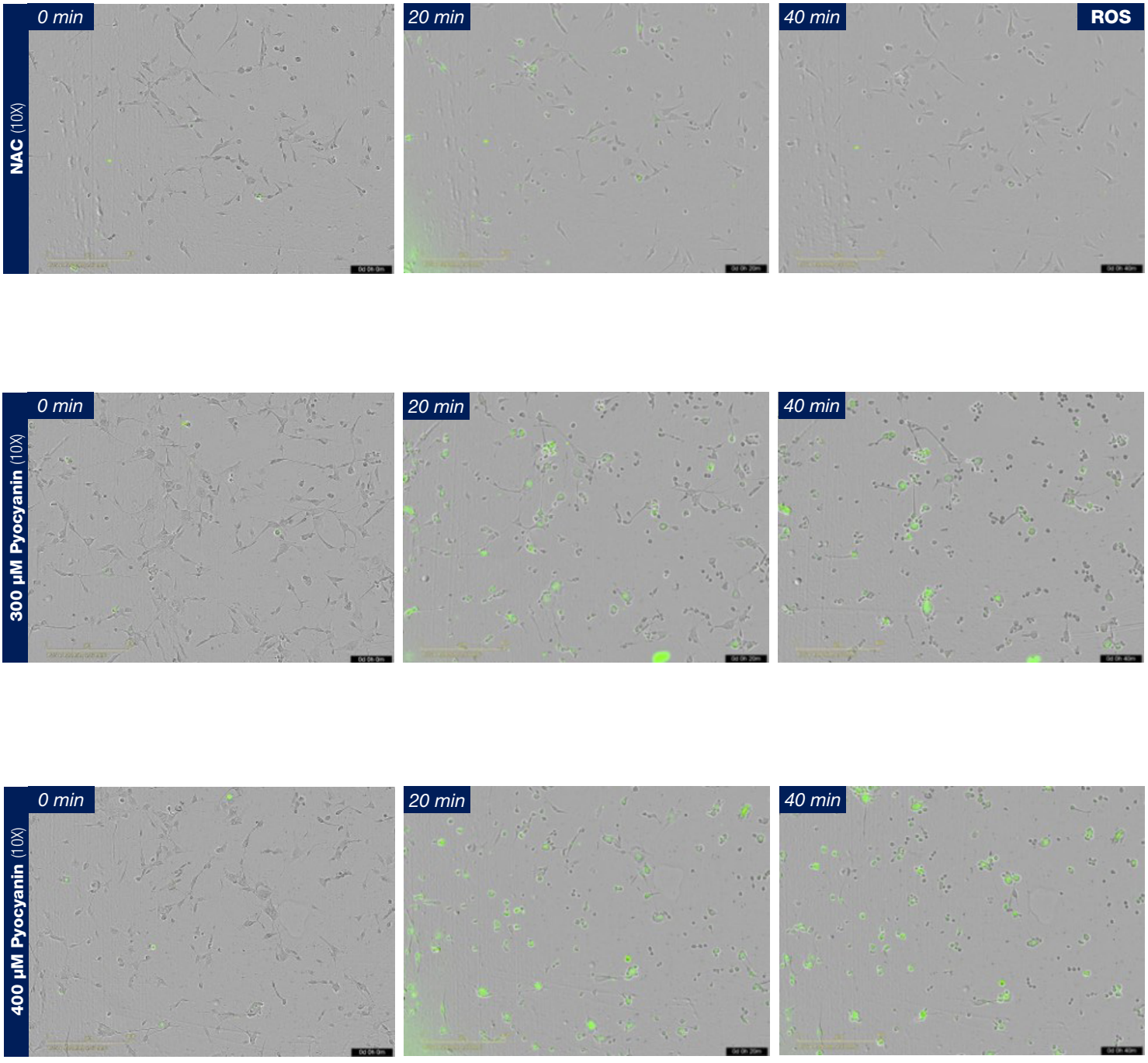


FIGURE 2: Aggresome detection in A549 cells. a) Fluorescent images of vehicle vs 5µM and 10µM MG-132 treated (18 hours) A549 cells. b) Representation of image segmentation performed by Operetta CLS™ to determine quantification in 2c and 2d. c) Quantification of images in 2a by the number of aggresomes per nuclei detected in the field of view. d) Quantification of images in 2a by total aggresome spot area per nuclei detected in the field of view.

3a.


0 min
20 min
40 min
ROS
NAC (10X)
0 min
20 min
40 min
300 µM Pyocyanin (10X)
0 min
20 min
40 min
400 µM Pyocyanin (10X)

3b.

3c.

3d.

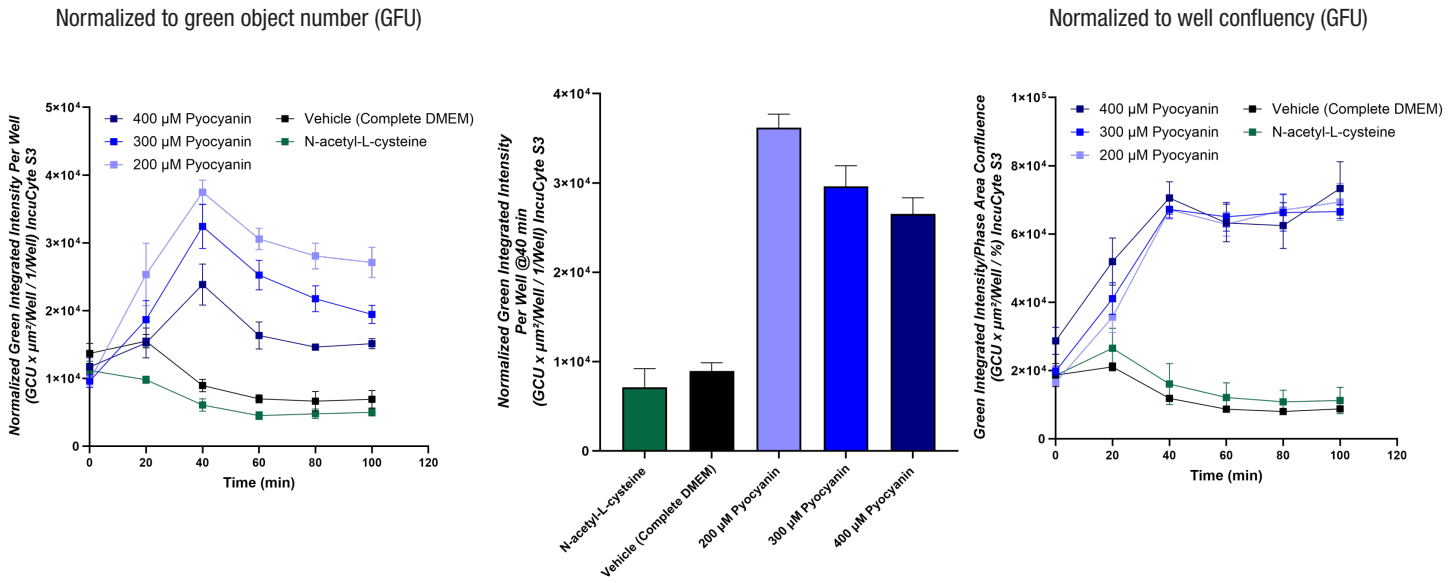
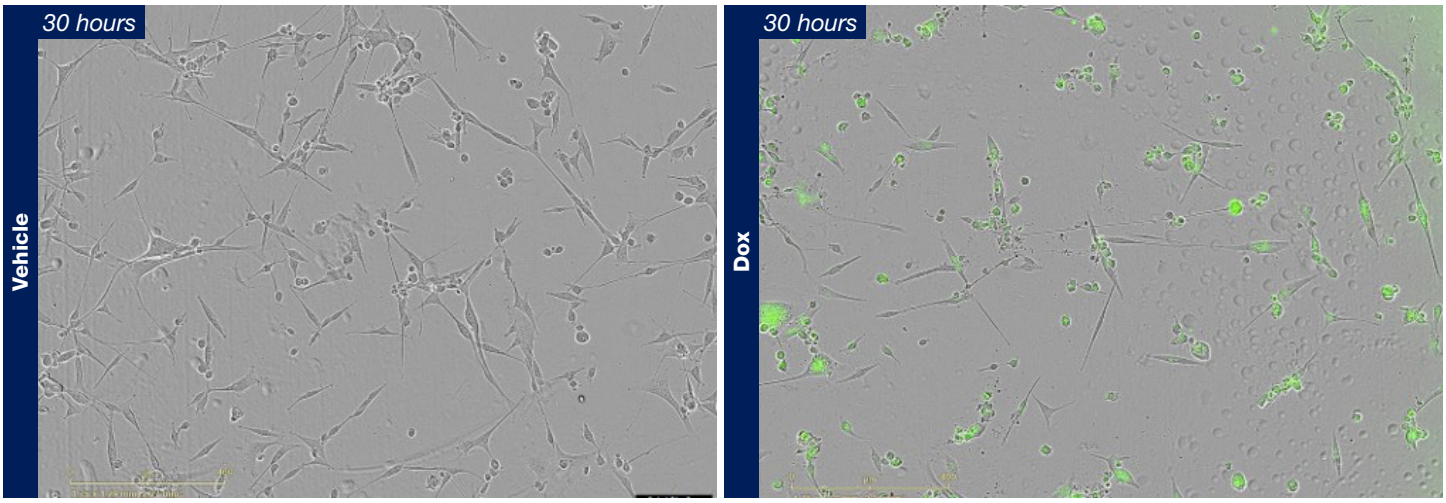


FIGURE 3: a) Brightfield images overlapped with fluorescent images of U87MG cells at 0-, 40-, and 60-minutes post treatment with either 5µM N-acetyl-L-cysteine (NAC) or 300µM pyocyanin with oxidative stress detection reagent. b) Time course graph of green intensity per object count for control and pyocyanin-treated cells. c) Values for 40-minute timepoint from 3b. d) Time course graph of green intensity per phase area confluence in control and pyocyanin-treated cells.

4a.



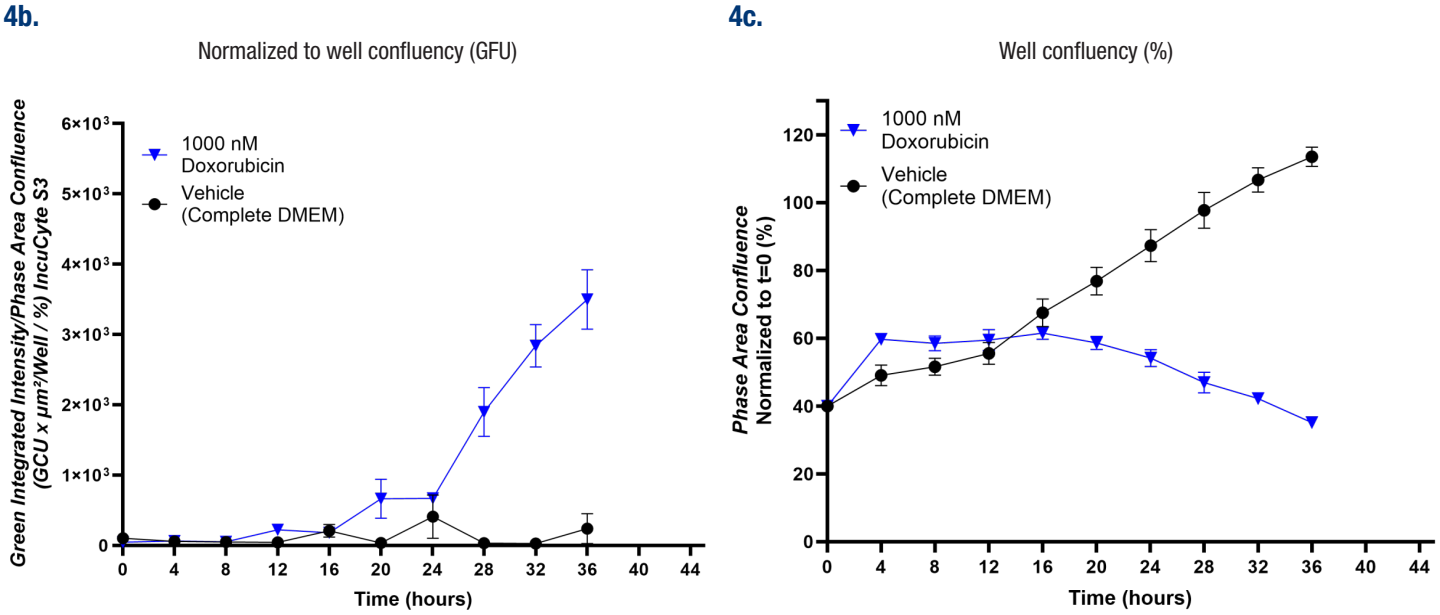
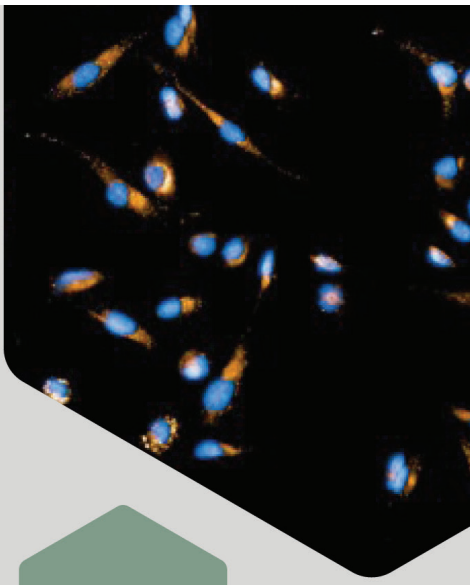


FIGURE 4: ROS detection in U87MG cells (Doxorubicin). a) Fluorescent images of control vs doxorubicin treated U87MG cells with oxidative stress detection reagent. b) Time course graph of green intensity per object count for control and doxorubicin treated cells. c) Time course graph of well confluency for control and doxorubicin treated cells.

DISCUSSION

Both the PROTEOSTAT® Aggresome Detection Kit and ROS-ID® Total ROS Detection Kit proved to be effective in analyzing the U87MG glioblastoma cell line. Furthermore, the PROTEOSTAT® Aggresome Detection Kit was effective in analyzing A549 carcinoma cell line. This highlights the versatility and utility of both kits with multiple cell types. Specifically, glioblastoma and carcinoma cell lines were validated through fluorescent imaging analysis on the IncuCyte® S3 and Operetta CLS™ automated platforms. These cell lines show the range of capabilities of both these two Enzo kits and the respective imaging platforms, as they feature classic star-convex morphology and irregular shapes for image segmentation. The results shown are consistent with various normalization methods for both endpoint and live cell imaging, and represent the exceptional performance of the assay controls included with each kit. This also highlights the compatibility of Enzo's live cell dye portfolio with multiple viable and robust tools for identifying, analyzing, and monitoring cellular activities in cell culture systems. Furthermore, these high-throughput and high-content screening methods open a wide range of possibilities for other potential research applications.



REFERENCES

For more information, visit enzo.com/neuroscience

Global Headquarters
ENZO LIFE SCIENCES, INC.
21 Executive Blvd.
Farmingdale, NY 11735
Ph: 800.942.0430
Fax: 631.694.7501
info-usa@enzolifesciences.com

European Sales Office
ENZO LIFE SCIENCES (ELS) AG
Industriestrasse 17
CH-4415 Lausen, Switzerland
Ph: +41 61 926 8989
Fax: +41 61 926 8979
info-eu@enzolifesciences.com

LOCAL EUROPEAN OFFICES

Belgium, The Netherlands & Luxembourg
Enzo Life Sciences BVBA
Avenue Louise 65/Box 11
1050 Bruxelles
Belgium
Ph: +32 3 466 0420
Fax: +32 3 808 7033
info-be@enzolifesciences.com

France
Enzo Life Sciences (ELS) AG
Branch Office Lyon
43, quai Perrache
69002 Lyon, France
Ph: +33 472 440 655
Fax: +33 481 680 254
info-fr@enzolifesciences.com

Germany
Enzo Life Sciences GmbH
Weiler Strasse 5e
DE-79540 Lörrach
Germany
Ph: +49 7621 5500 526
Fax: +49 7621 5500 527
info-de@enzolifesciences.com

UK & Ireland
Enzo Life Sciences (UK) Ltd.
1 Colleton Crescent
Exeter EX2 4DG
Ph: 0845 601 1488 (UK customers)
Ph: +44 1392 825900
Fax: +44 1392 825910
info-uk@enzolifesciences.com

For local distributors and detailed product information visit us online:
www.enzo.com