Cellular analysis

Biotherapeutic lysosomal degradation pathway illuminated by LysoLight dye staining and real-time monitoring with the EVOS M7000 Imaging System with OSI-2

Introduction

Antibody–drug conjugates (ADCs) are therapeutic agents that are pivotal in targeted cancer therapy. An ADC consists of an antibody coupled with a toxic payload, such as a chemotherapy drug. The monoclonal antibody component of an ADC selectively targets cancer cells by binding to a specific antigen on their surface, which allows for specific delivery of the drug payload to cancer cells while minimizing damage to healthy cells. Drug efficacy of an ADC relies on its precise delivery and release within cancer cells, specifically within the lysosomes, to exert a cytotoxic effect. For an ADC to undergo catabolism in the lysosome, it must bind to the cell surface and be internalized through the endocytic pathway. However, the binding of an antibody to a target cell does not invariably lead to its internalization or catabolism since it can bypass the lysosome and enter the recycling pathway instead. Therefore, when developing biologic-based therapies for cancer treatment, it may be necessary to evaluate the cellular localization and ultimately catabolic fate of the therapeutic agent.

Measuring the catabolic fate of antibodies and other proteins

Fluorescent tools like pH-sensitive dyes can be used to monitor internalization of antibodies into endocytic pathways. However, measuring the catabolic fate of internalized molecules remains time-consuming, requiring low-throughput and semiquantitative methods like immunoblotting. We developed Invitrogen™ LysoLight™ Deep Red and LysoLight™ Green fluorogenic dyes to address the challenges of determining the catabolic fate of

internalized molecules. Unlike pH-sensitive dyes, LysoLight dye fluorescence is controlled by a cathepsin-cleavable linker so that it only fluoresces when it enters the lysosome and is cleaved by the lysosome-specific enzyme cathepsin. LysoLight Deep Red and Green dyes are easily conjugated to antibodies or other proteins with solvent-exposed lysine residues using Invitrogen™ LysoLight™ Antibody Labeling kits. LysoLight dyes are sensitive and nontoxic, allowing for live-cell imaging experiments over multiple days. We paired the Invitrogen™ EVOS™ M7000 Imaging System with the second-generation EVOS™ Onstage Incubator (OSI-2) to demonstrate the utility of this system for monitoring the internalization and degradation of the targeted cancer drug trastuzumab conjugated to LysoLight Green dye in SKBR3 cells, a model for HER2-positive breast cancer, over the course of 3 days. Trastuzumab is a therapeutic monoclonal antibody that specifically targets the overexpressed HER2 receptor in certain breast cancer cells, and it is used alone and as the antibody component of ADCs to treat HER2-positive breast cancer.

Materials

Product	Supplier	Cat. No.
SKBR3 cell line	ATCC	HTB-30
Trastuzumab	Bio X Cell	SIM0005
McCoy's 5A (Modified) Medium	Thermo Fisher Scientific	16600082
LysoLight Green Antibody Labeling Kit	Thermo Fisher Scientific	L36005
EVOS M7000 Imaging System	Thermo Fisher Scientific	AMF7000
EVOS Onstage Incubator (OSI-2)	Thermo Fisher Scientific	AMC2000

Protocol

LysoLight dye conjugation to trastuzumab

LysoLight Deep Red and LysoLight Green dyes are amine reactive, so they are easily conjugated to antibodies or other proteins that have solvent-exposed lysine residues. The following is a general procedure for the conjugation of LysoLight Green dye to trastuzumab using the LysoLight Green Antibody Labeling Kit for labeling 100 µg of antibody. For more details, please see the LysoLight Green Antibody Labeling Kit user guide.

1. Add 1 mL of ultrapure water to the supplied vial of sodium bicarbonate to make a 1 M stock solution.
2. Prepare 80 µL of trastuzumab at a concentration of 1.25 mg/mL using the supplied 1X PBS buffer.
3. Add 10 µL of 1 M sodium bicarbonate to the trastuzumab antibody prepared in step 2.
4. Add 10 µL of the supplied DMSO to one vial of the LysoLight Green amine-reactive dye and vortex to fully dissolve.
5. Add the trastuzumab antibody mix prepared in step 3 to the vial of LysoLight Green dye prepared in step 4 and gently vortex, then spin briefly to collect the sample at the bottom of the vial.
6. Incubate the reaction mixture in the dark for 4 hours at 25°C with gentle shaking.
7. Following the conjugation reaction, purify the LysoLight Green dye–labeled trastuzumab using the supplied Thermo Scientific™ Zeba™ Dye and Biotin Removal column, pre-equilibrated with PBS exchange buffer.
8. Measure the absorbance of the purified conjugate at 280 nm and 496 nm to calculate the antibody concentration and degree of labeling (DOL).

Determination of exposure settings for the live-cell time-lapse experiment

LysoLight Green dye is nonfluorescent until it is cleaved by lysosomal cathepsins. Therefore, LysoLight Green dye must be fully cleaved to achieve signal saturation to determine exposure settings on the EVOS M7000 Imaging System. The following is a detailed protocol describing how to saturate the signal to determine the exposure settings using internalization and degradation of trastuzumab conjugated to LysoLight Green dye in SKBR3 cells. If using another model system, the time to reach maximum signal intensity will entirely depend on your particular cell line and antibody.

1. Grow SKBR3 cells to 80% confluency in McCoy's complete medium.
2. Seed 100 µL of the cells in a 96-well tissue culture plate at a density of 5,000 cells/well.
3. Allow the cells to recover for at least 6 hours.
4. Dilute LysoLight Green dye–labeled trastuzumab to 10 µg/mL in McCoy's complete medium and add 10 µL/well to give a final concentration of 0.91 µg/mL.
5. Incubate for 24 hours (or until maximum signal intensity is reached) in a tissue culture incubator set to 37°C, 5% CO_2, and 80% relative humidity.
6. Image the wells containing the internalized LysoLight Green dye–labeled trastuzumab on the EVOS M7000 Imaging System.
   a. Adjust the light intensity, gain, and exposure settings and ensure that the signal is not saturated.
   b. Record these settings and use for 72-hour time-lapse imaging.

72-hour time-lapse imaging on the EVOS M7000 Imaging System with OSI-2

The following are details on how to set up the EVOS M7000 Imaging System with OSI-2 for live-cell imaging of the degradation of LysoLight Green dye–labeled trastuzumab in SKBR3 cells. The EVOS OSI-2 can be used to perform time-lapse imaging for 72 hours by providing optimal conditions for cell growth while significantly reducing moisture buildup. Moisture buildup limited the original EVOS OSI to a maximum of 24 hours of time-lapse imaging.

1. Prepare the SKBR3 cells as described previously and seed at a density of 5,000 cells/well.
2. Prepare the EVOS OSI-2:
   a. Fill the water tray to the mark with purified water.
   b. Connect the CO_2 lines.
   c. Turn on the incubator with the EVOS M7000 software and ensure that the incubator settings are adjusted to 37°C, 5% CO_2, and 80% relative humidity, then allow the settings to stabilize. It is recommended to perform this step at least 30 minutes before starting the experiment.
3. Place cells on the EVOS M7000 Imaging System and adjust the automation settings (see the EVOS M7000 Imaging System manual for detailed instructions):
   a. Hardware settings:
      i. Vessel type: 96-well plate
      ii. Objective: 20x
      iii. Channel: GFP and transmitted
      iv. Exposure settings: use the light intensity, exposure, and gain settings that were previously determined
   b. Scan area: locate an appropriate field of view
   c. Autofocus: enable and autofocus on transmitted signal
   d. Time-lapse and incubator: set the imaging frequency and length of time
   e. Image save settings: select destination folder
4. After configuring the automation settings, add the 10 µg/mL LysoLight Green dye–labeled trastuzumab to the cells and start the time-lapse experiment.
5. Perform data processing with EVOS or ImageJ software.

Results from a live-cell imaging experiment are shown in Figure 1.

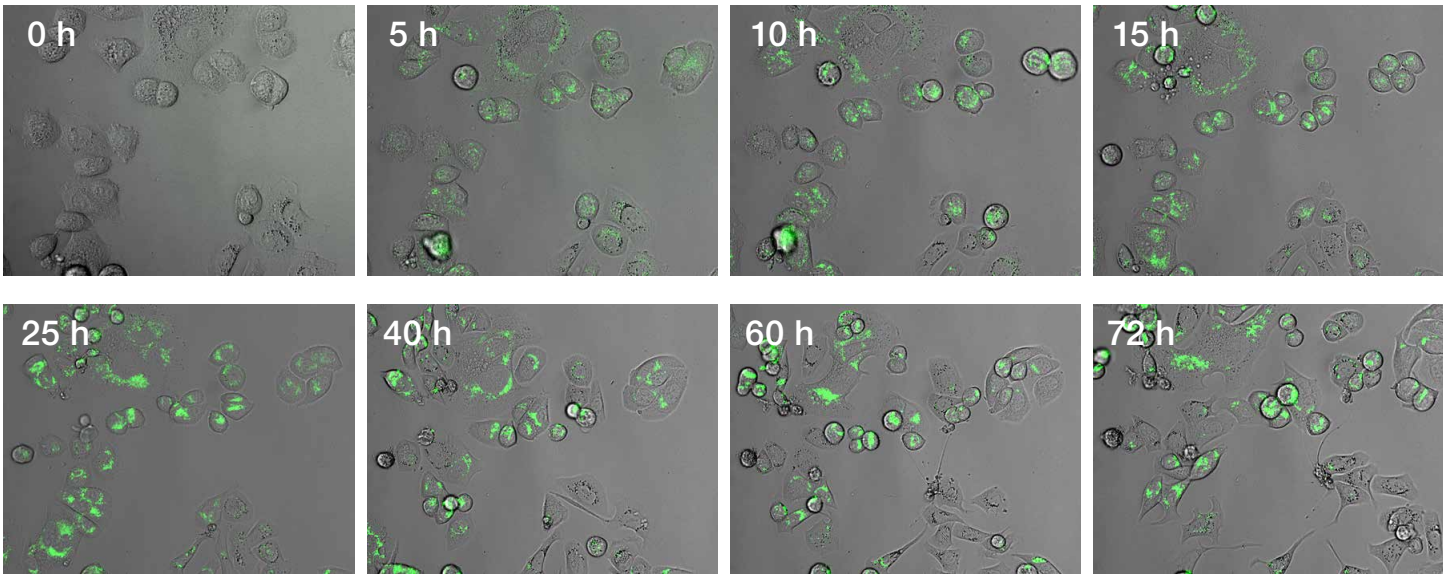

Figure 1. Time-lapse imaging of the degradation of LysoLight Green dye–labeled trastuzumab in live cells. SKBR3 cells were treated with 1 µg/mL of LysoLight Green dye–labeled trastuzumab for 72 hours. Images were taken every 15 minutes on the EVOS M7000 Imaging System with OSI-2. The signal increased as the conjugate of LysoLight Green dye and trastuzumab degraded, with signal intensity beginning to plateau around 24 hours. While no toxicity measurements were performed, cells were actively dividing throughout the 72-hour experiment.

Conclusions

LysoLight Deep Red and Green dyes are first-in-class sensors that enable specific and sensitive monitoring of antibody degradation. To demonstrate this, we conjugated LysoLight Green dye to the monoclonal antibody–based cancer drug trastuzumab and incubated the conjugate with SKBR3 cells to perform time-lapse imaging. SKBR3 cells are a model for HER2-positive breast cancer cells since they are positive for HER2 receptors, while trastuzumab binds to HER2 receptors and is used to treat HER2-positive cancers. LysoLight Green dye–conjugated trastuzumab showed initial signal generation within the first 3 hours of incubation with SKBR3 cells, then the signal plateaued over 24 hours. Importantly, the LysoLight Green dye signal was stable for 72 hours, and the dye was nontoxic to the cells.

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