Use of High content imaging to enable Cell-based Potency assay development for ADCs

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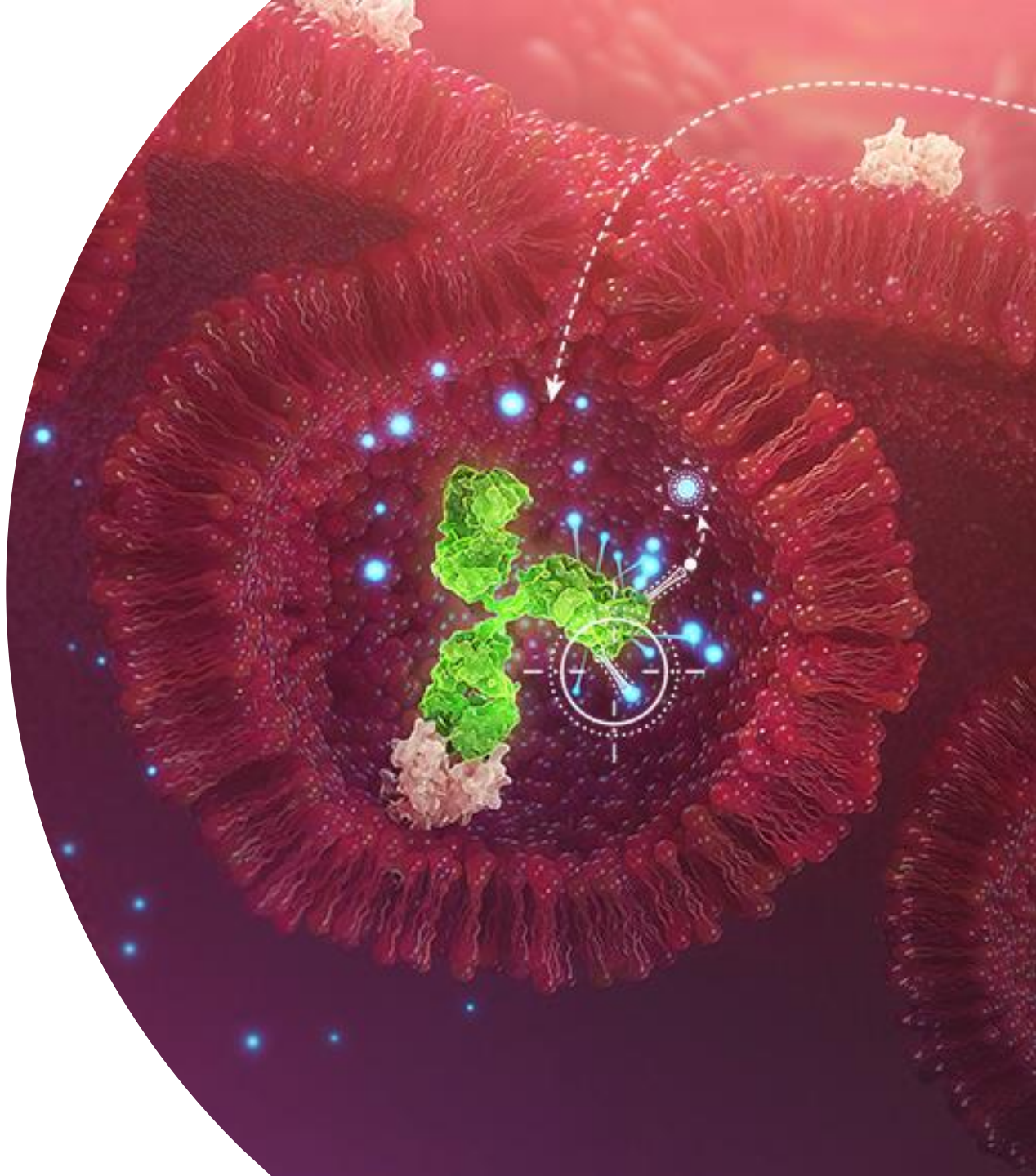
CASSS Bioassays

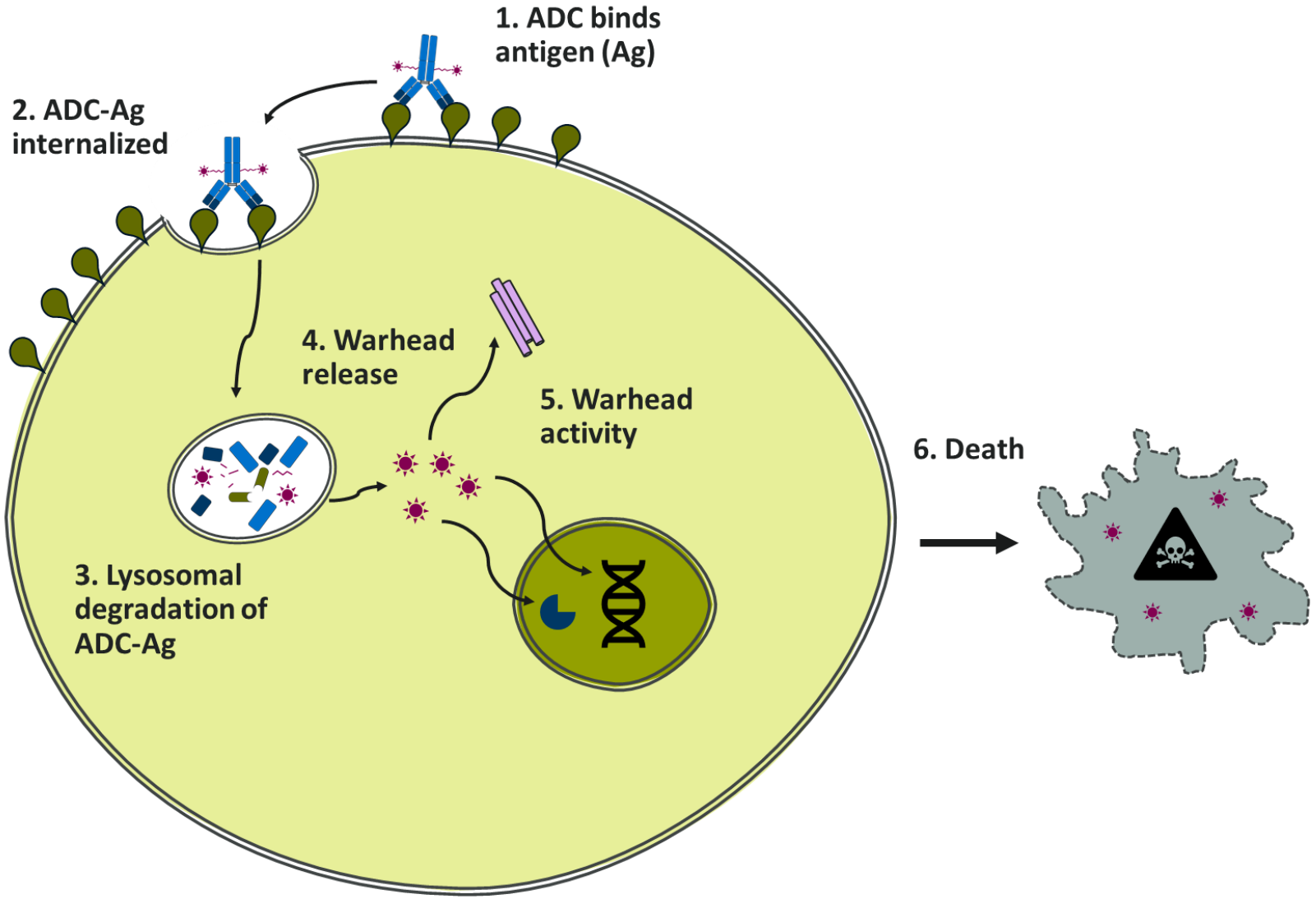
Session III-ADCs **April 09, 2025**



A biological missile…

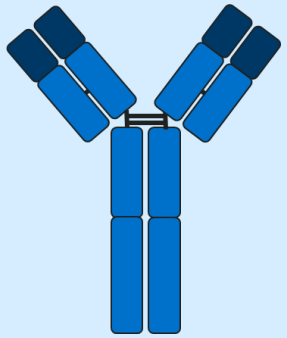
1. MOA
2. CMC strategies for potency assays for ADCs
3. Challenges in developing potency assays in the current ADC landscape
4. IncuCyte as a work-horse to enable cell-based potency assay development for ADCs
5. Using the IncuCyte characterization of biological activity
6. Concluding remarks

Primary ADC MoA is payload delivery / warhead-induced cytotoxicity

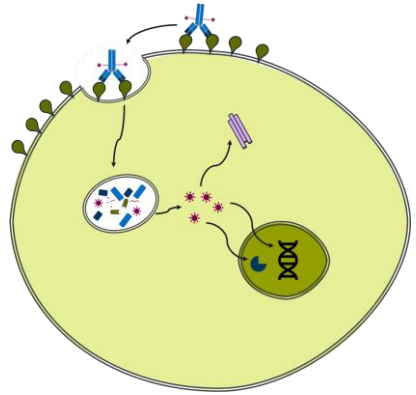


Standard GMP potency assays for ADCs

mAb intermediate

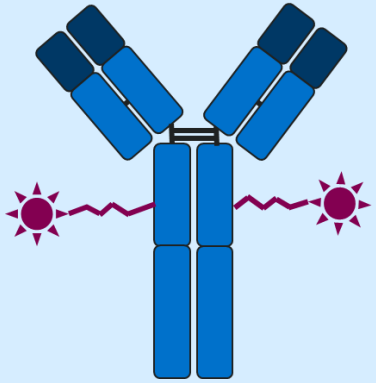


1. **Target-antigen binding**
   - Cell or non-cell based
   - Ensures potency before conjugation
   - Common methods: ELISA
   - Common readouts: Fluorescence or colorimetric



ADC

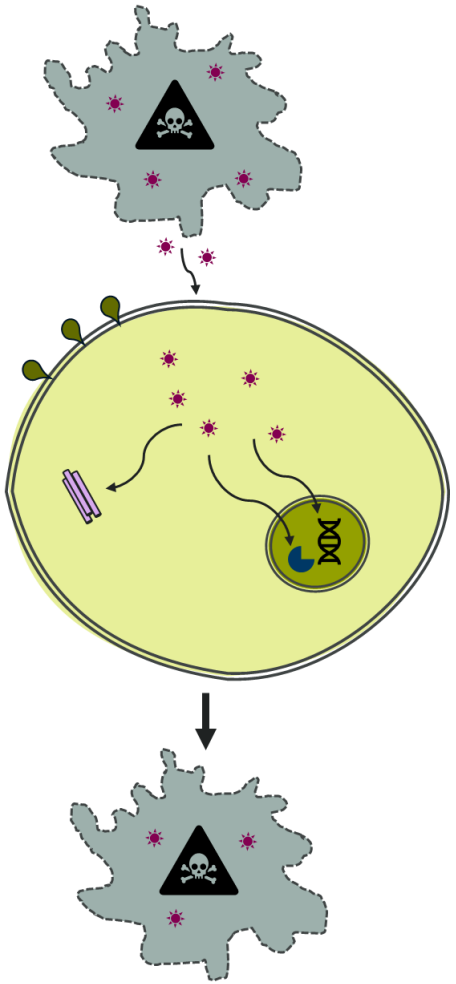
(DS and DP)



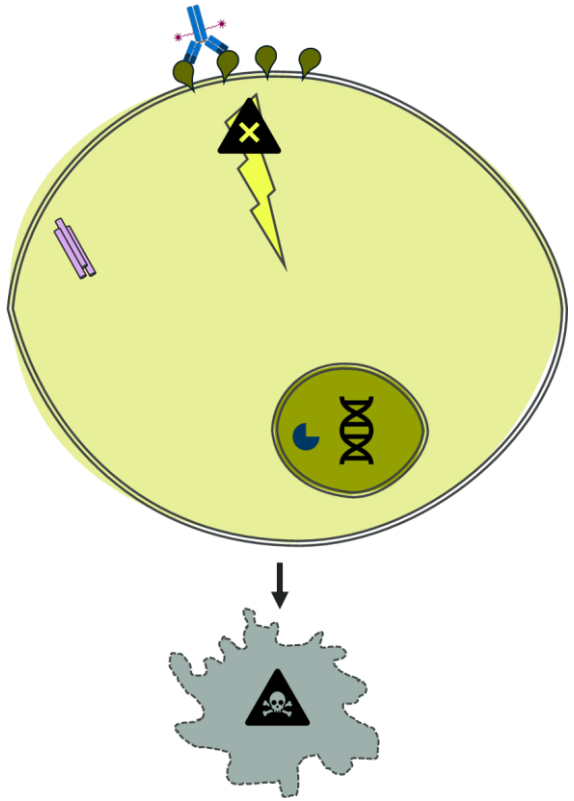
1. **Target-antigen binding**
   - Identical assay as mAb intermediate
   - Ensures conjugation does not impact target binding
2. **Cytotoxicity assay**
   - Cell-based
   - Common endpoints: reduction in ATP production, membrane integrity
   - Common readout: Luminescence or colorimetric or fluorescence

ADCs can possess secondary MoAs

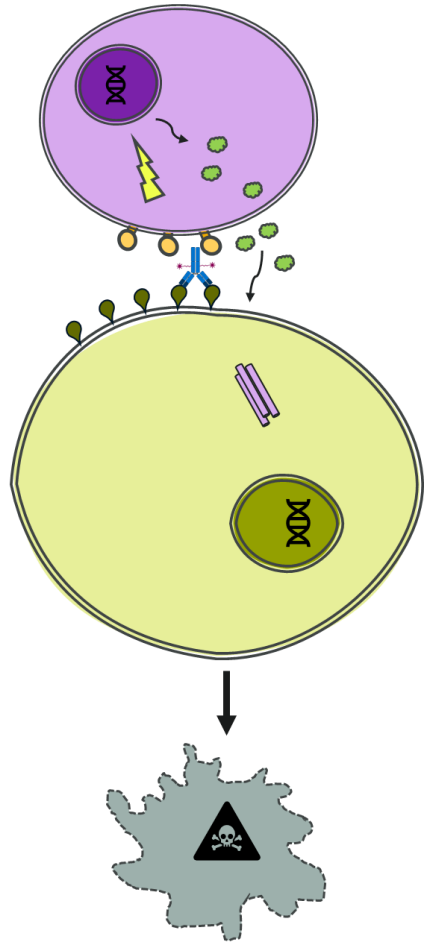
1. Bystander effect



2. Antigen signaling inhibition



3. Fc-effector functions



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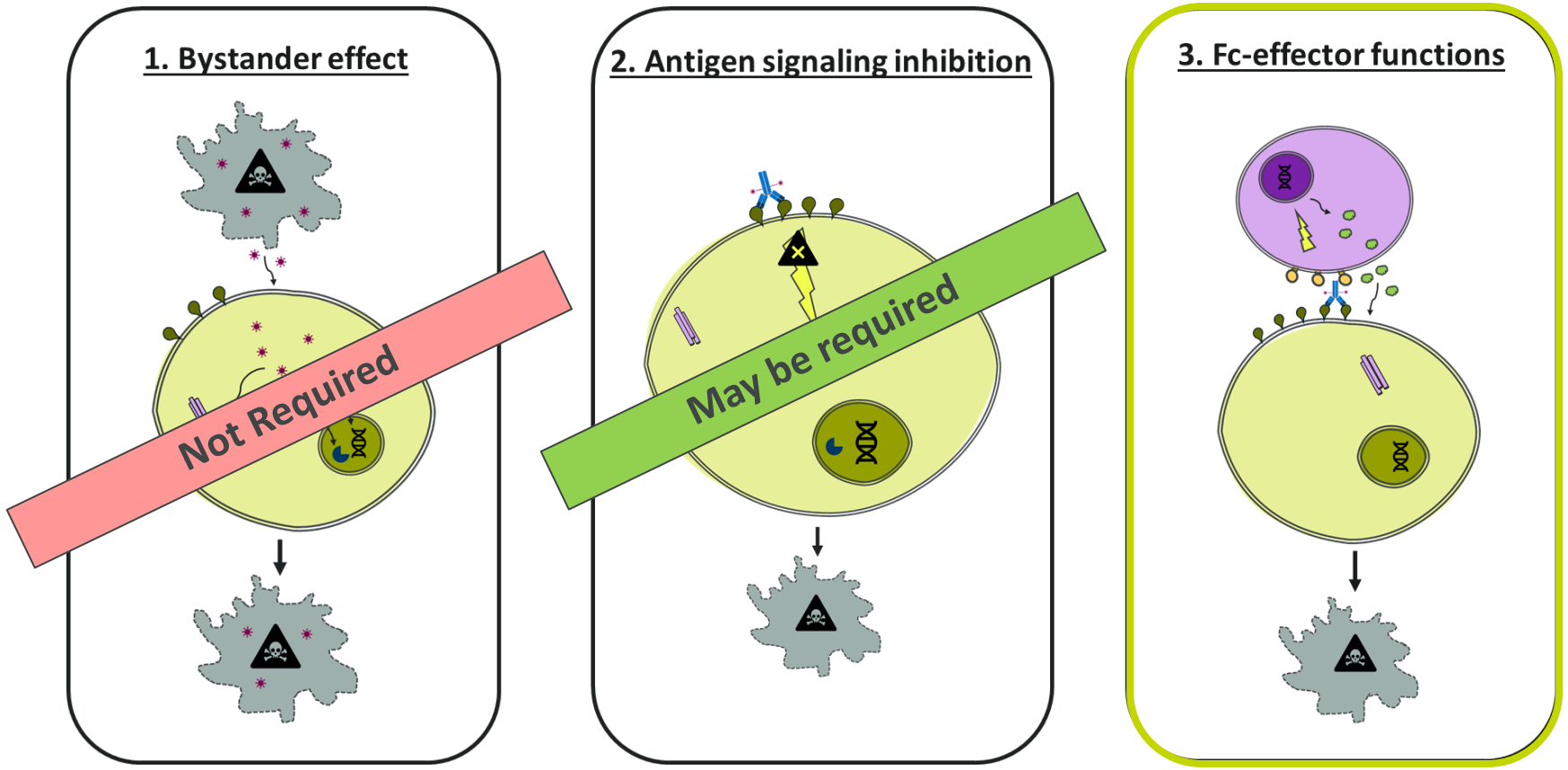
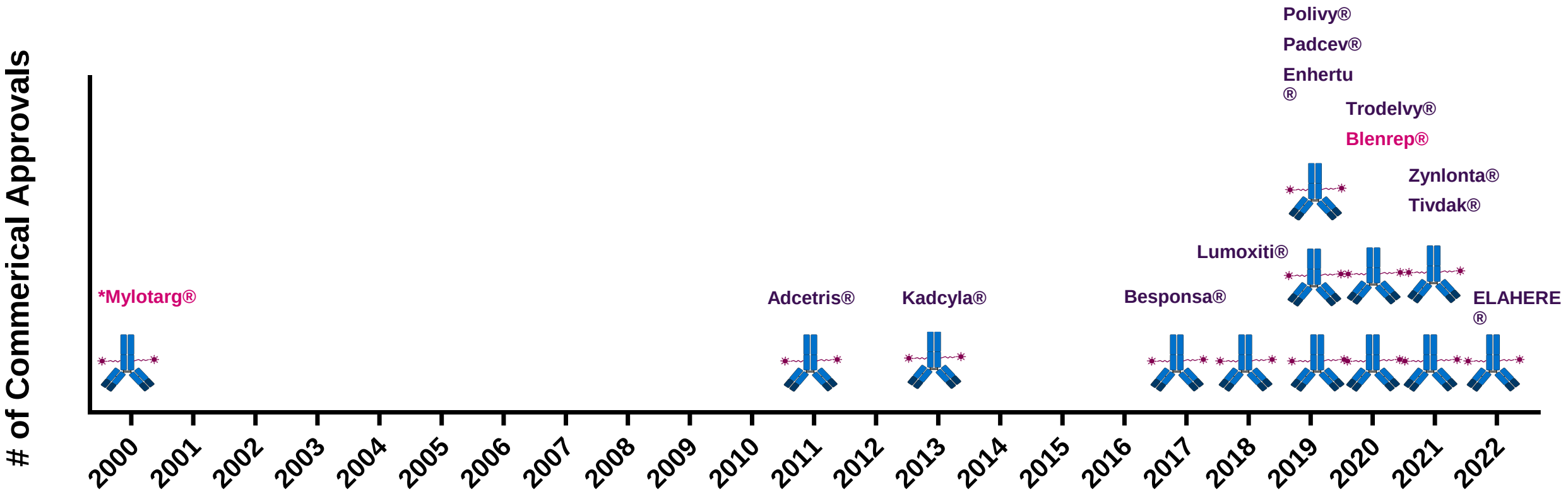


Control may be required

- Mechanisms should be characterized at early phase.
  → ADCC, CDC, and ADCP
- If activity detected, CMC control is expected (characterization assay).
  → Cell-based functional assays, FcyR binding, and/or N-linked oligosaccharide profile

Frequency and number of commercial approvals is only recently on the rise

13 Commercial Approvals to Date



Red = withdrawn

*re-approved in 2017

Next generation ADCs and beyond….

New conjugation chemistries

- Site-specific: Inserted cysteines or unnatural AA's
- Branched linkers: [Anami et al., (2017) Ang Chem Inter 56:733-737]
- Non-covalent conjugations: [Gupta et al., (2019) Nat Biom Engin 3:917-929]

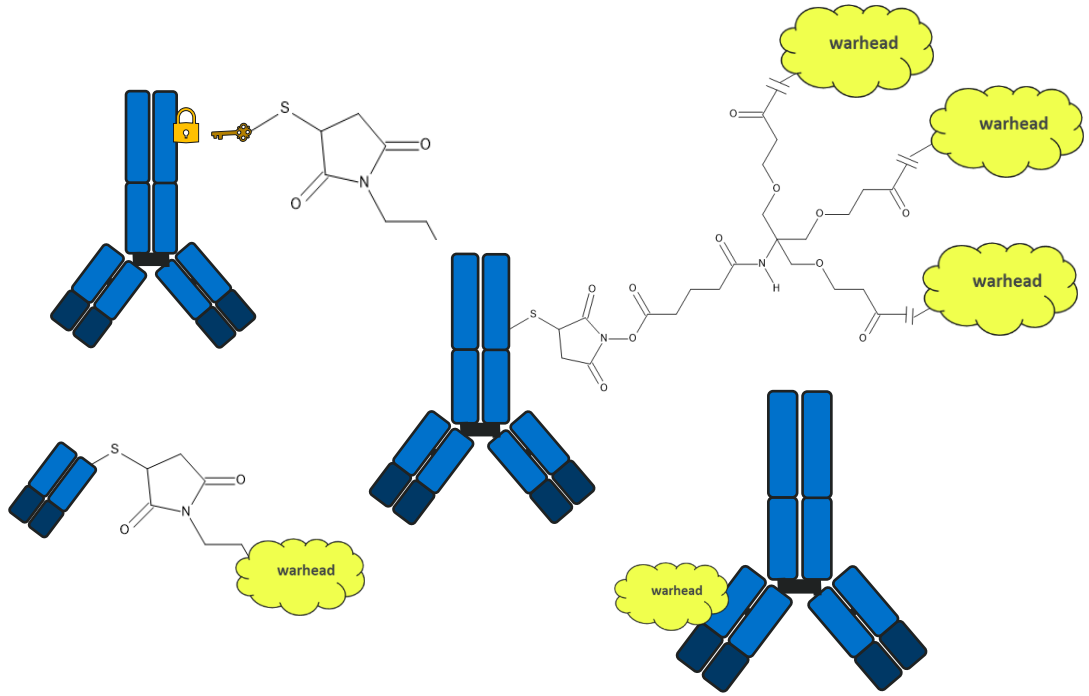
Non-traditional antibody formats

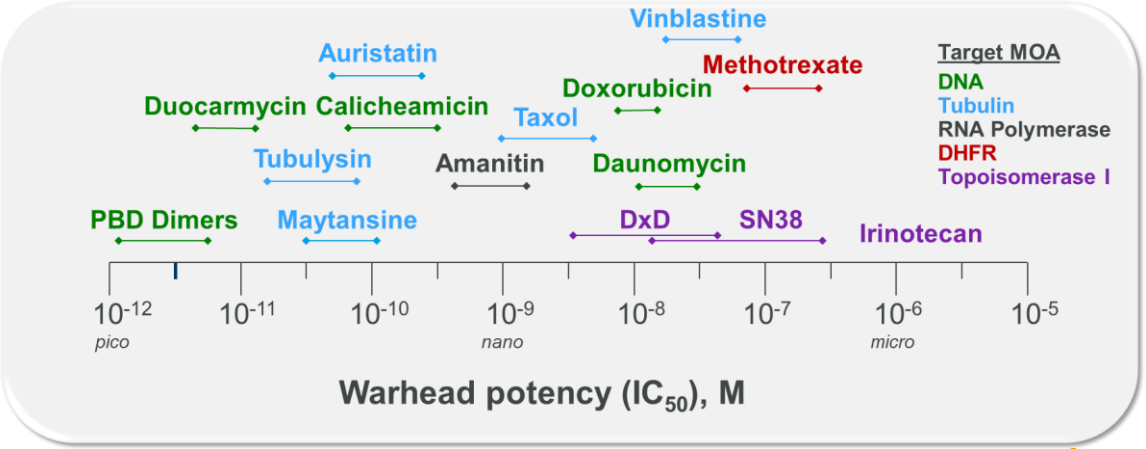
Growing toolbox of warheads

- Greater diversity of MoAs, trigger different pathways to cell death
- Impact of DAR # and DAR distribution to cytotoxicity and effector function
- Wide range of potencies
- Multi-warhead conjugates

More selective target antigens for cancer or tumor microenvironment

Enabling new and accelerated ADC pipelines requires outside-the-box strategies, methodologies, and technologies for CMC bioassay





Using the IncuCyte as a work-horse to develop Cytotoxicity Assays

Live cell imaging: IncuCyte (Sartorius)

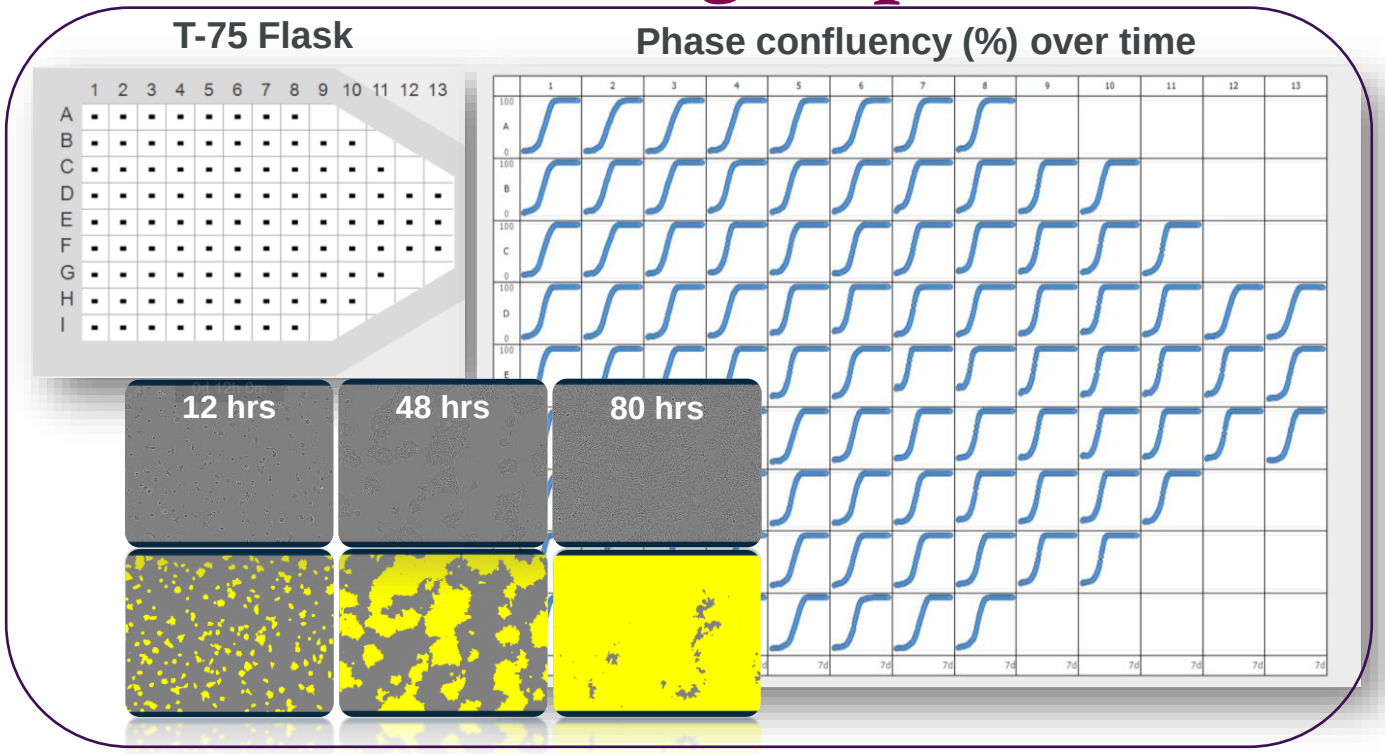
Phase and fluorescence high content imager contained within a 37°C incubator

- Continuous monitoring and analysis, allows for early detection and decision making
- Near real-time visualization and automated analysis of living, non-perturbed cells
- Up to 5 fluorescent channels allowing imaging of fluorescently labelled cells (SX5)
- Improves the ability to support newer modalities working under aggressive timelines

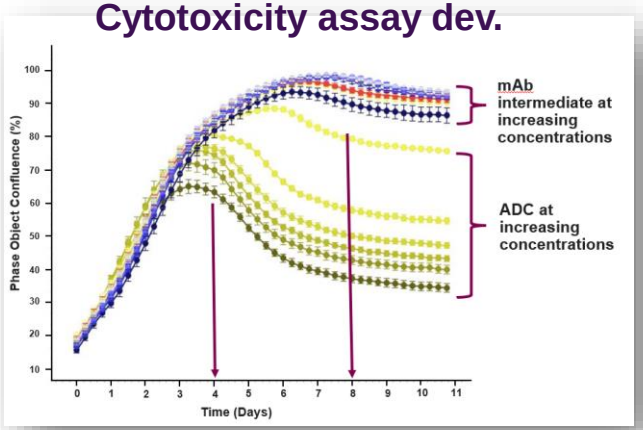
Live cell imaging: IncuCyte current uses in our group

In-vitro characterization

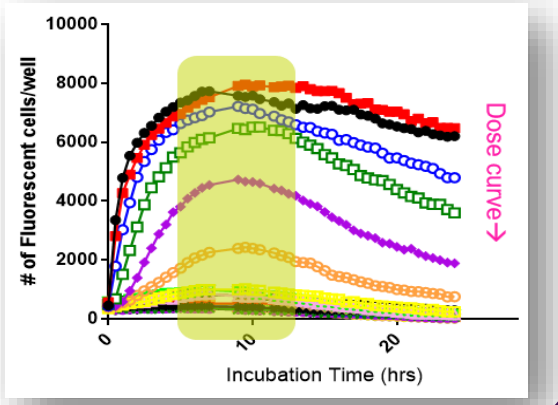
- Cell line screening
- Cell seeding density optimization
- Spheroid growth
- Cytotox assay development
- Fc effector function: ADCC, CDC characterization
- mAb internalization studies
- ADC bystander effect- ongoing effort



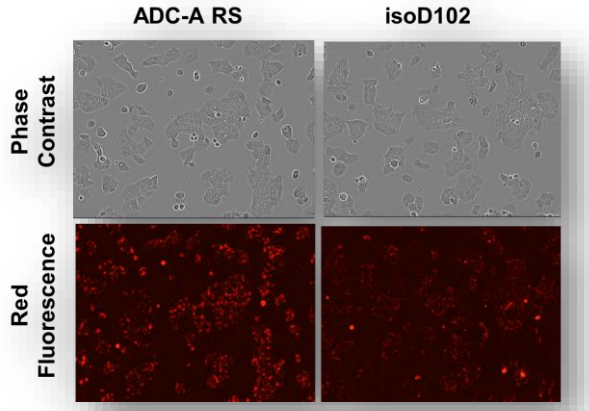
Cytotoxicity assay dev.



CDC assay dev.



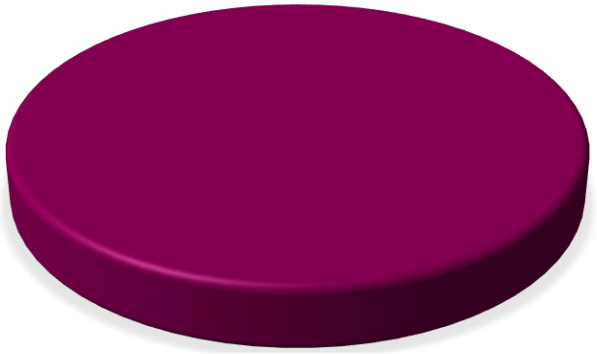
mAb internalization



Finding the correct cell line for the Cytotox assay can be a challenge

Cytotoxicity by Cell-Titer Glo

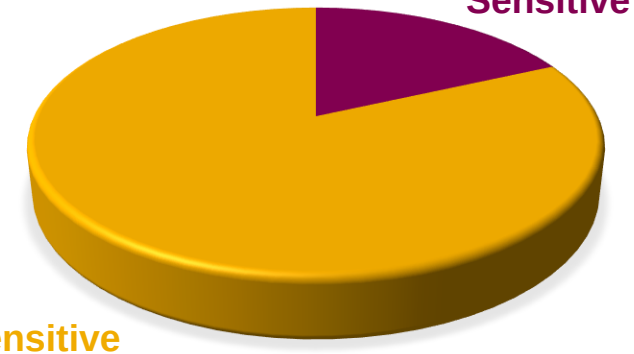
ADC 1.1 (WARHEAD A, DAR2)



Sensitive

11 (of 11) cell lines

ADC 1.2 (WARHEAD B, DAR8)

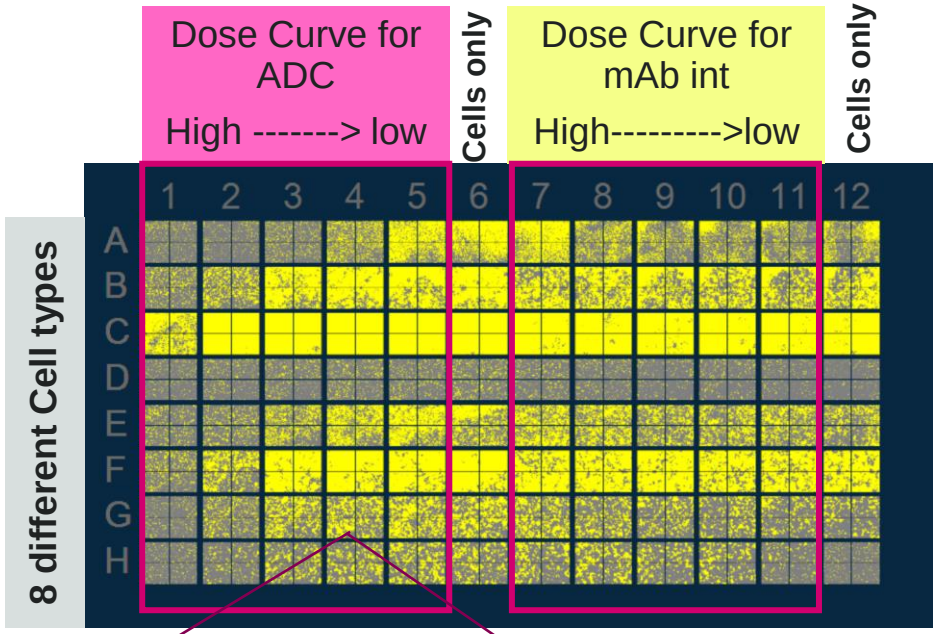


Sensitive 2 (of 11) cell lines

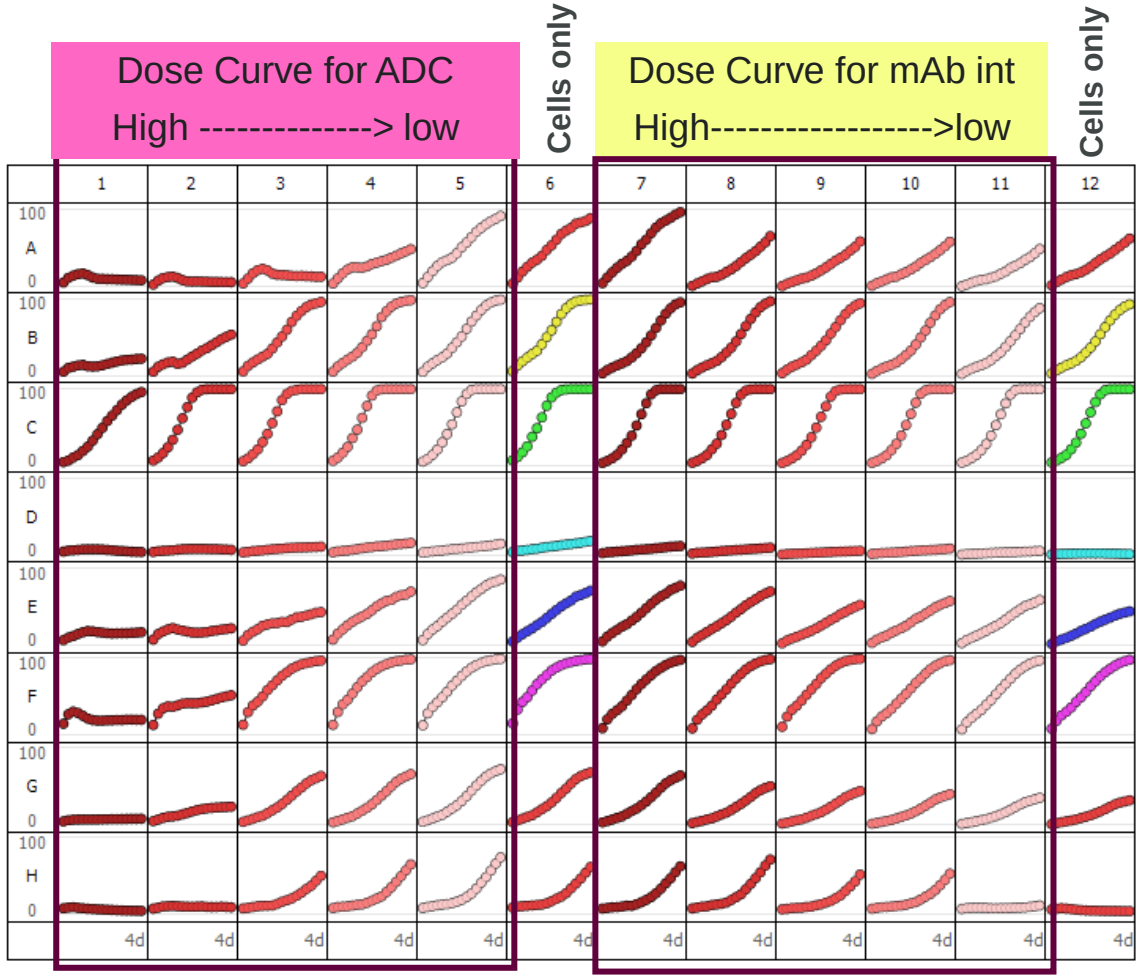
Insensitive

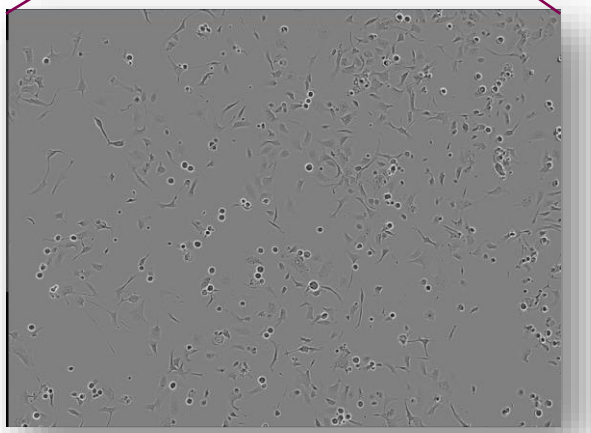
- ADCs directed against the same target antigen
- Different warheads with different MOAs and different DAR distribution
- Lack of sensitivity could be due to the warhead's susceptibility to neutral-basic pH
- Screening each cell line using traditional methods in a cytotox assay that takes 4+ days to run would take too long

Example of cell line screening for Cytotoxicity Assays using a 96-well plate in the IncuCyte

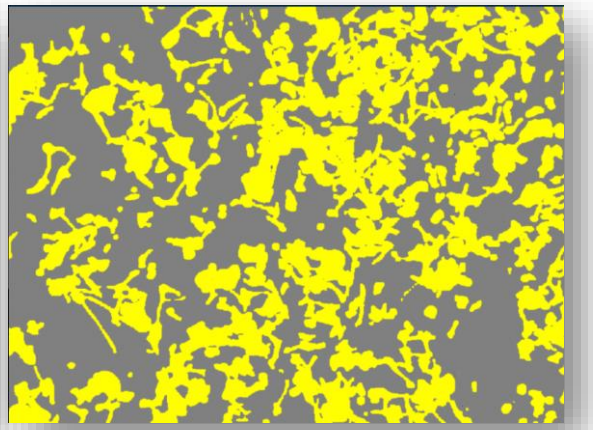


Analysis





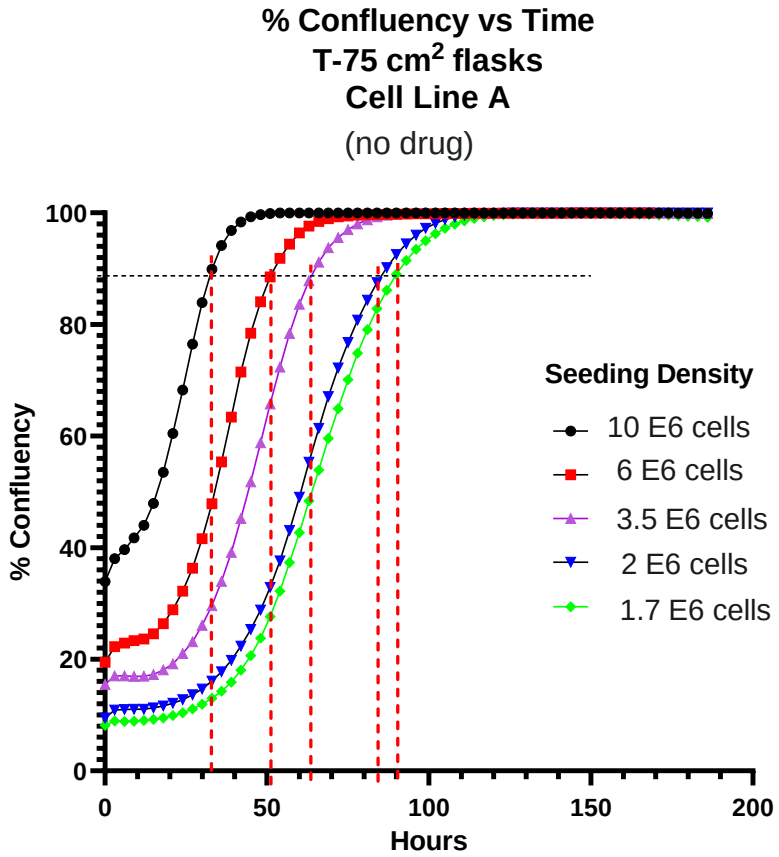
Phase Contrast image of confluency

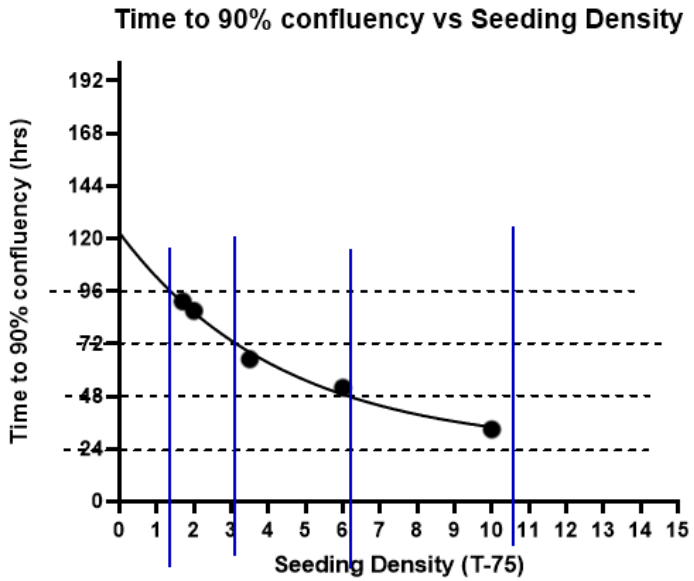


Masking by software to count confluency

Enables dye free screening of cell lines

Cell culture condition optimization using the IncuCyte





Days in Culture	Projected Seeding density Flask size (cm^2) (example)	
	T-75 flask	T-175 flask
1	Est. 10.5	Est 24.5
2	6.2	14.5
3	3.1	7.2
4	1.3	3.0

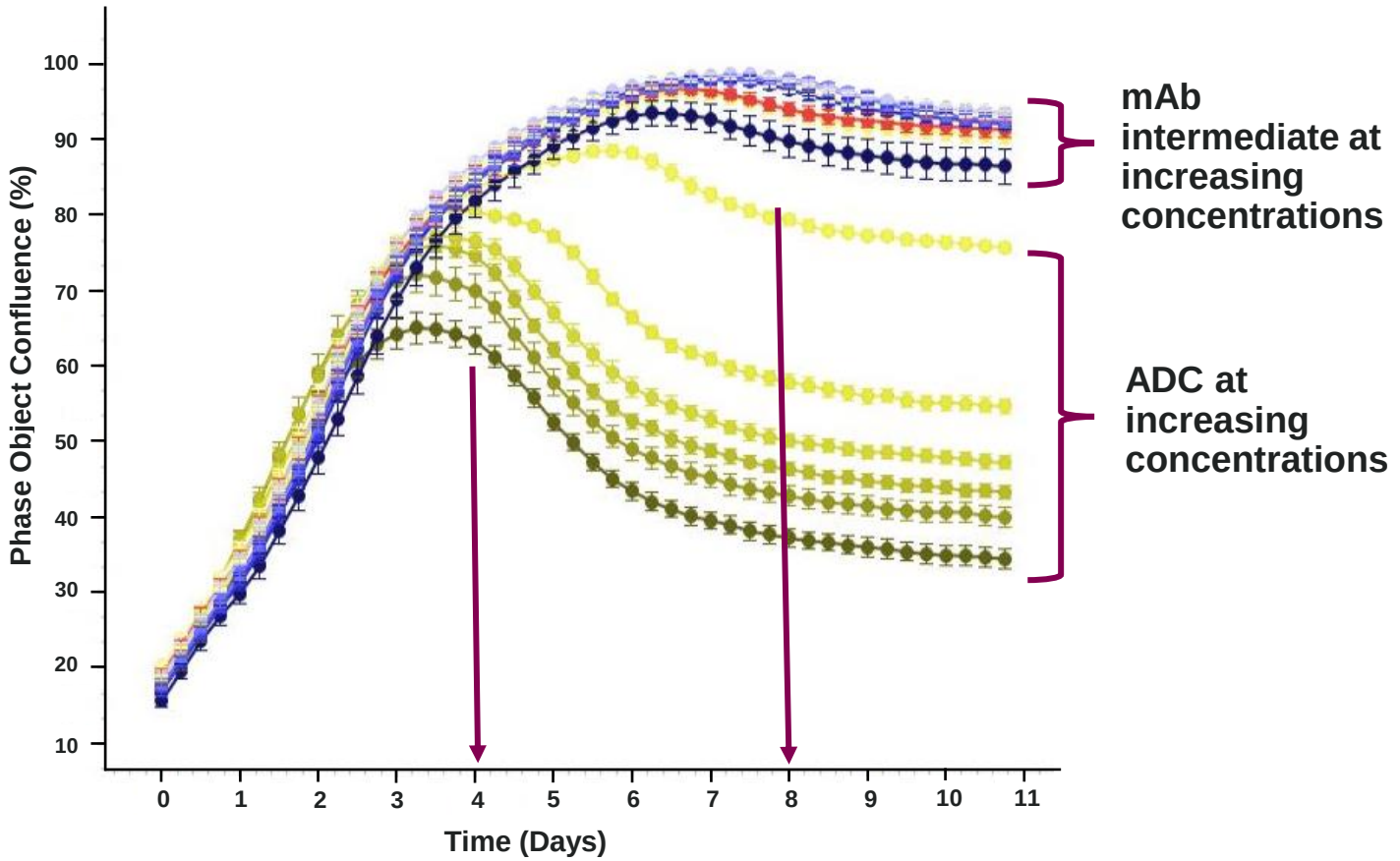
Once a cell line has been selected….

- Label-free confluency analysis
- Perform objective and quantitative analysis of confluency
- Leave cells in the IncuCyte and walk away…
- Extrapolate data to find optimum seeding densities and passaging time
- Gives accurate estimates of doubling rates for cell lines with no prior experience

Cytotoxicity Assay development
Preliminary scouting experiment using the IncuCyte

In this experiment…

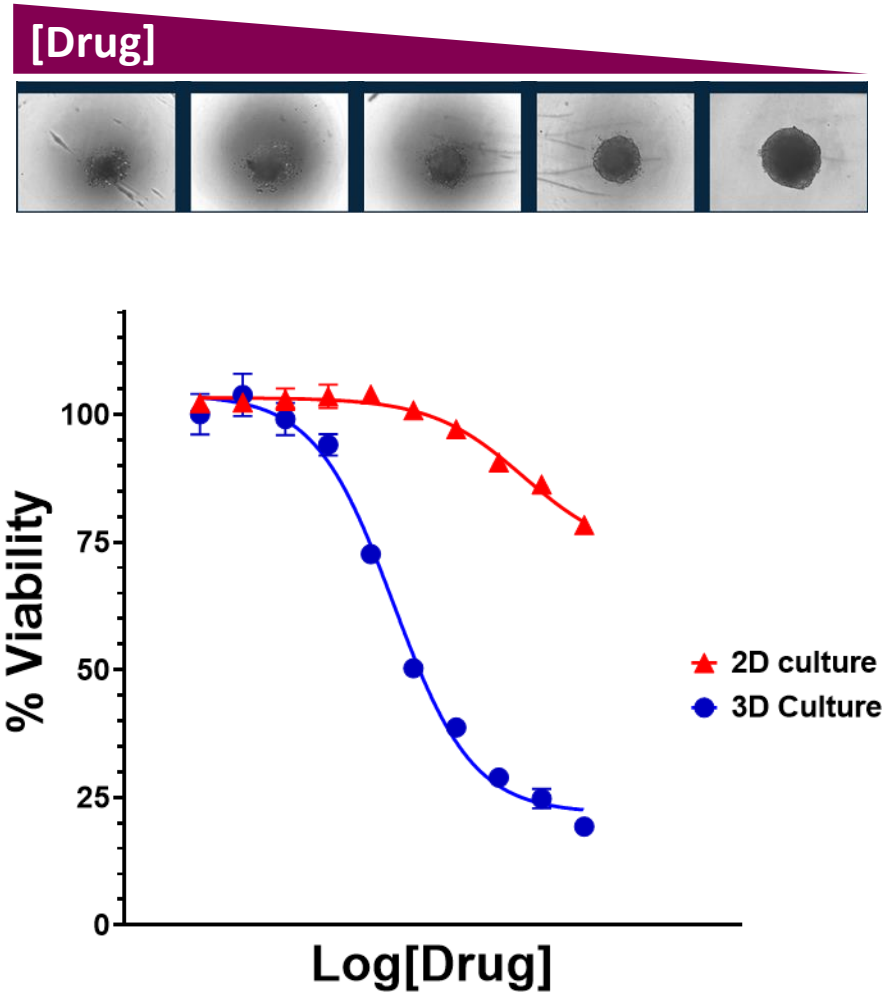
- Start to see killing around Day 4
- Plateaus around Day 8
- Can vary depending on warheads, DAR ratios
- Helps to determine optimal assay window
- Live cell analysis vs end-point analysis during assay development work



Spheroid culture increases susceptibility to ADC-induced death

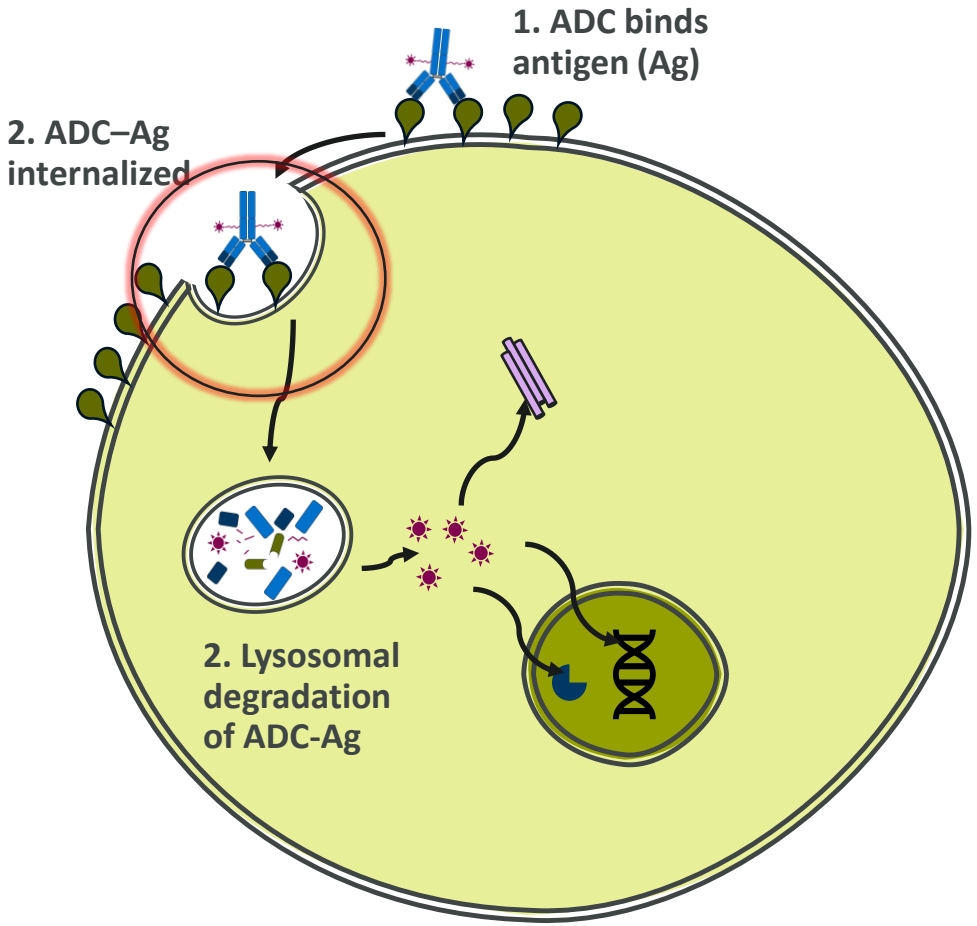
- **Growing evidence suggests that culturing cells in 3D better mimics in vivo properties**
  - Morphology
  - Genetic epigenetic regulation
  - Metabolism
  - Drug sensitivity and toxicity
- **Seeding cells in non-adherent, round-bottom assay plates most GMP friendly way to encourage spheroid formation**
- **Screening spheroid cultures becomes easy with the IncuCyte**
  - Can observe spheroids in 3D
  - Quantify time needed to form spheroids
  - Quantify time needed to kill

ADC- induced cell death

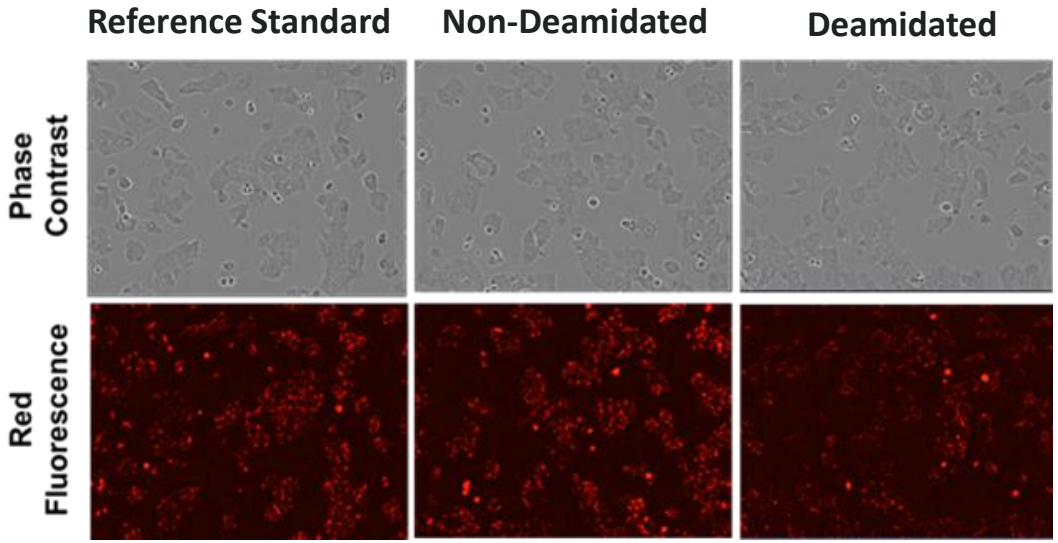


Biological Characterization Assessment using the IncuCyte

Investigating ADC internalization using the IncuCyte-enabling CQA evaluation



Impact of asparagine deamidation to antigen binding and subsequent ADC internalization



ADC-A samples were labeled with 5µ/gmL Incucyte® Human Fabfluor-pH Red antibody labeling dye, which is a pH sensitive red-fluorophore that only fluoresces in the low pH environment of a lysosome.

Imaged by Incucyte S3 fluorescent imager for phase contrast and red fluorescence under 20x objective every 20 min.

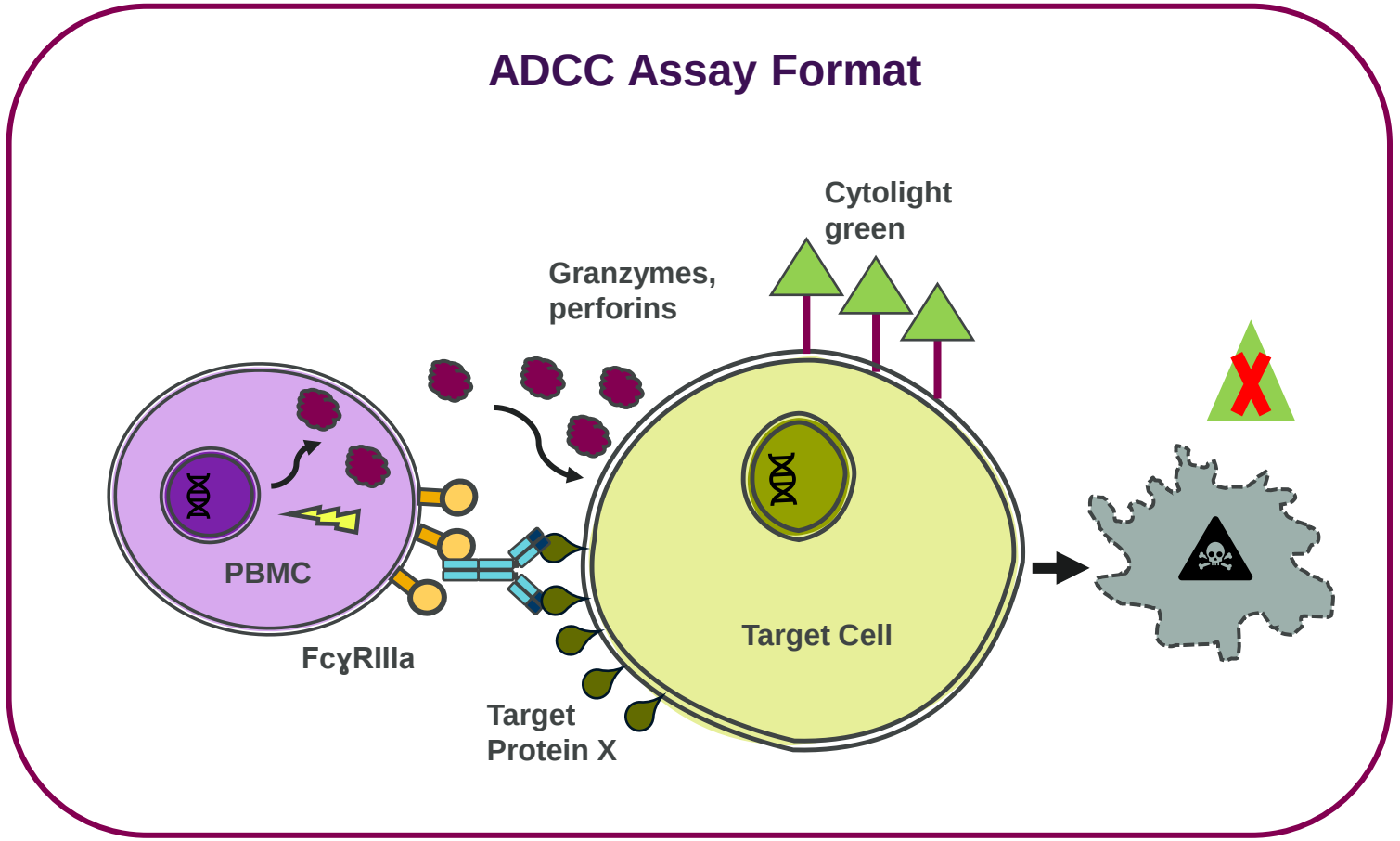
Cao M et al., (2023) Antibodies, 12(4):68

Fc Effector Function: *In vitro* ADCC assay development using the IncuCyte

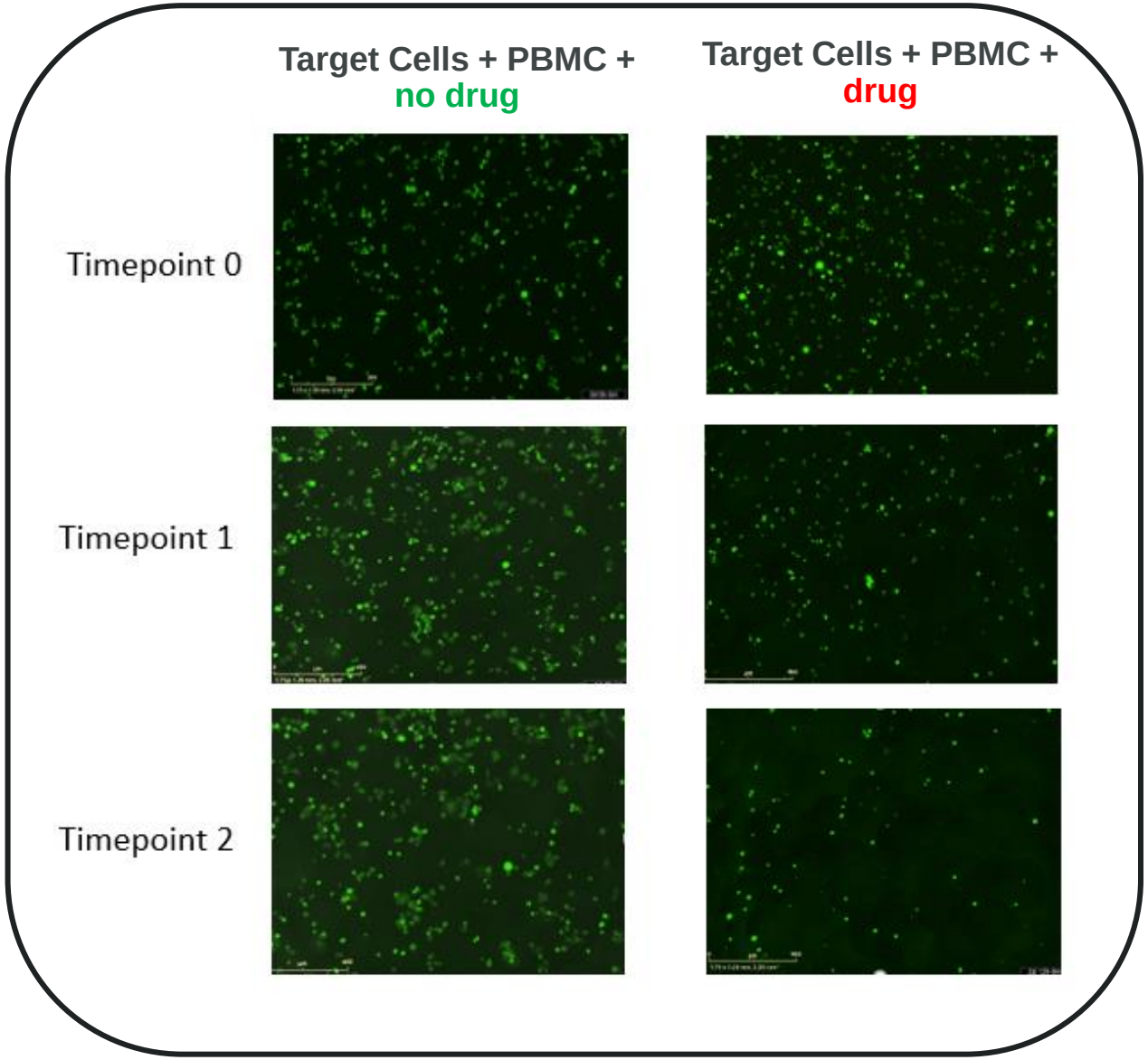
Label target cells with Cytolight green marker (live cell cytoplasmic labelling)

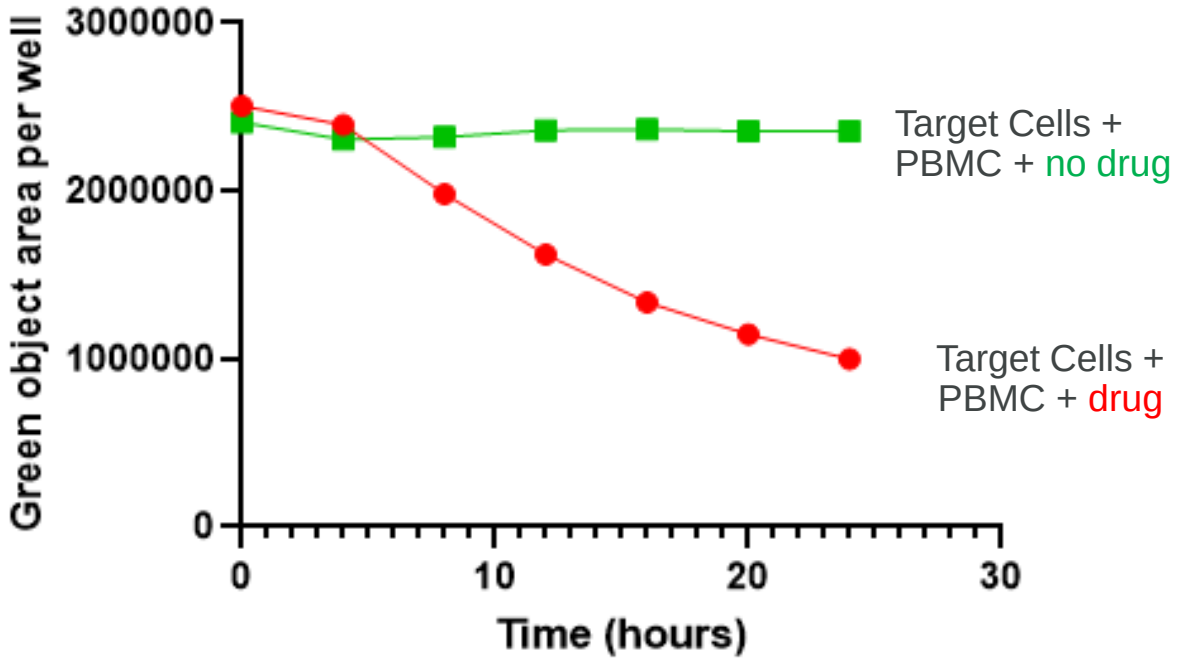
Target Cells+ effector cells+ Drug: Mix labelled target cells with freshly isolated PBMCs (effector cells) at 20:1 E:T ratio and add to assay plate with dose response curve of mAb

IncuCyte: Place 96-well plate into IncuCyte and perform live cell imaging. Real time visualization of target cell killing



In vitro ADCC activity determination using IncuCyte





Conclusions & Discussion

- **New ADCs hold great promise in the clinic, but pose unique challenges to CMC bioassay**
- **Growing number of warheads require robust toolbox of cytotoxicity methods**
  - Range of potencies and MoAs may require new endpoints and readouts
  - Potency and efficacy of some warheads are not well recapitulated in vivo
  - 3D culturing may overcome in vitro sensitivity challenges
- **Live Cell Imaging using the IncuCyte has greatly improved our efficiency in developing Cell-Based lot release potency assays, characterization assays**
  - Reduces time for screening appropriate cell line that are sensitive to killing by new warheads
  - Employed live cell-imaging to aid in cell line characterization, culture optimization, cytotox assay development work, Fc effector function characterization, CQA assessments

Acknowledgements

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Elizabeth Christian

Dylan Weil

Christina Grigoriadou

Scott Umlauf

Sartorius- IncuCyte Team

Lorenzo McMillan

Erin Weddle

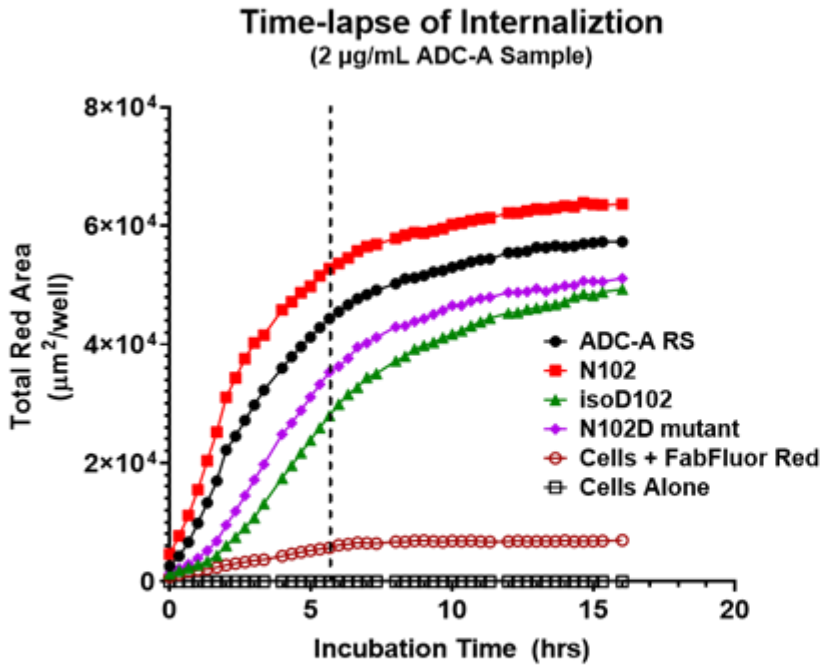
CASSS Bioassays Organizers

Thank
you!

BACK UP

Investigating ADC internalization using the IncuCyte-enabling CQA evaluation

A.



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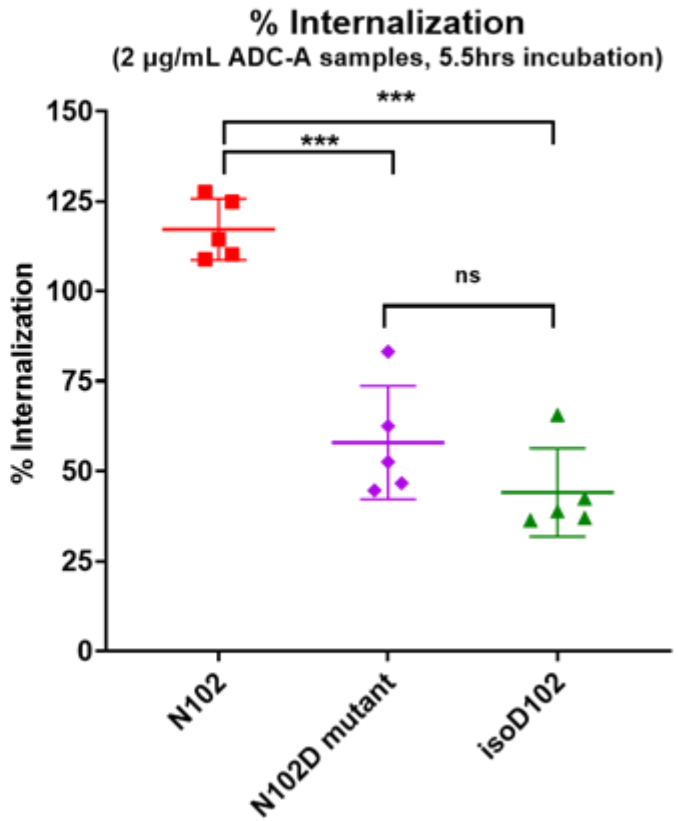

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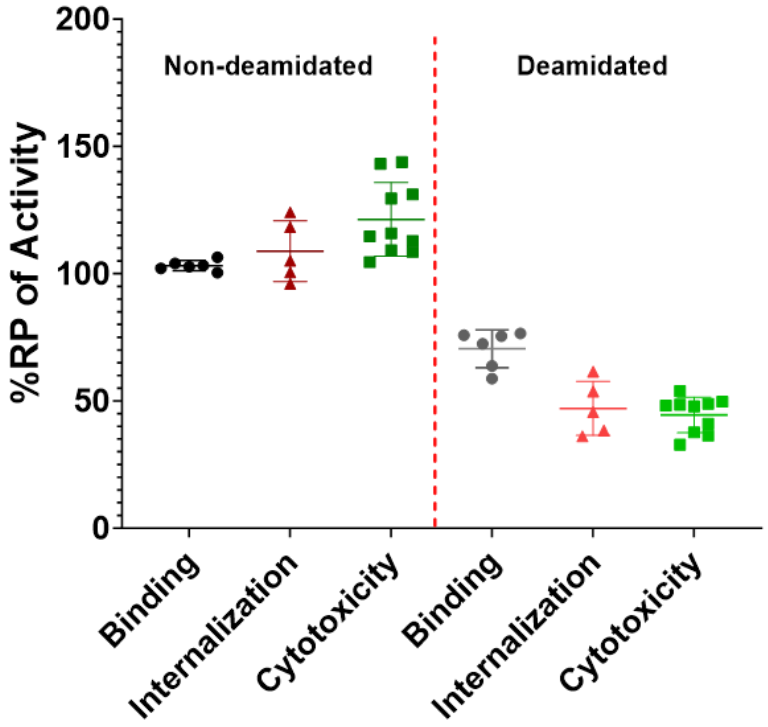


Cao M et al., (2023) Antibodies, 12(4):68

ADC-induced death in spheroids

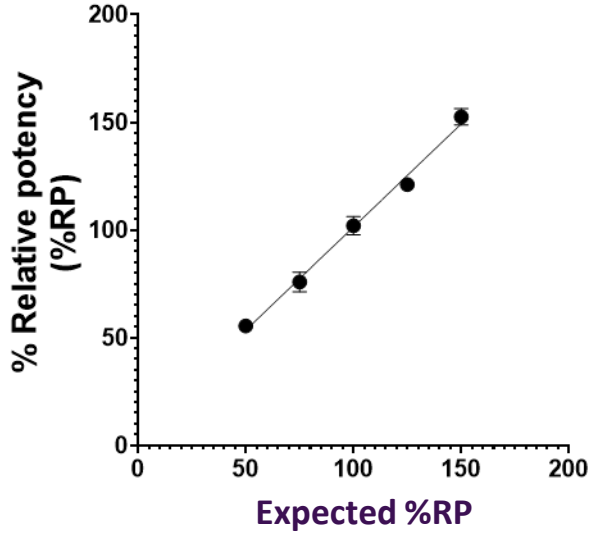
Spheroid-based cytotoxicity assay is…

- Accurate and precise within 50 – 150% RP range
- Stability-indicating
- Demonstrates acceptable performance for qualification and GMP-use

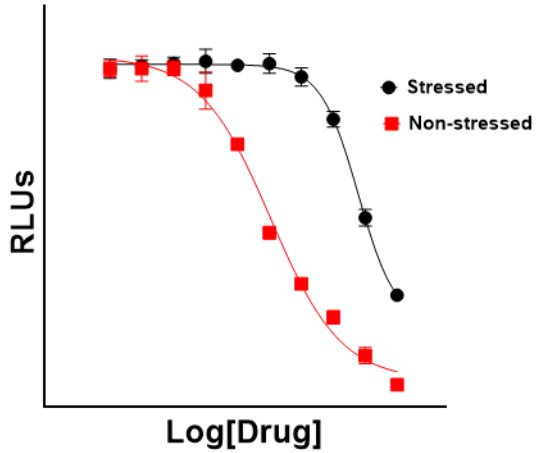
Mechanism of increased sensitivity not well-known, but could be due to…

- Increased antigen surface expression
- Syncing cell replication
- Inclusion of more cell-cell junctions and physiological morphology
- Creation of tumor microenvironment

Linearity



Stability-indicating



Next generation ADCs and beyond….keeps us on our toes!

Evolving conjugation strategies and growing toolbox of linkers and warheads are advancing ADCs through the clinic…

New conjugation chemistries

- Site-specific: Inserted cysteines or unnatural AA's
- Branched linkers: [Anami et al., (2017) Ang Chem Inter 56:733-737]
- Non-covalent conjugations: [Gupta et al., (2019) Nat Biom Engin 3:917-929]

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Growing toolbox of warheads

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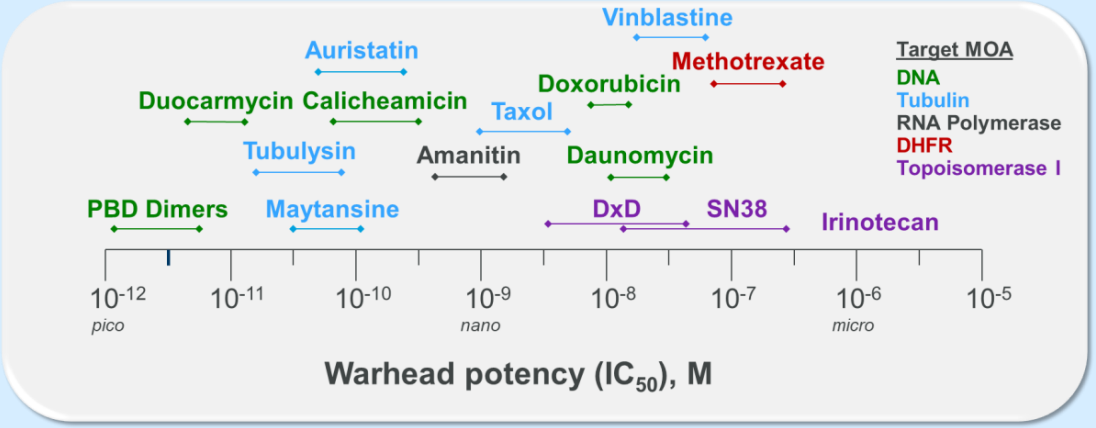
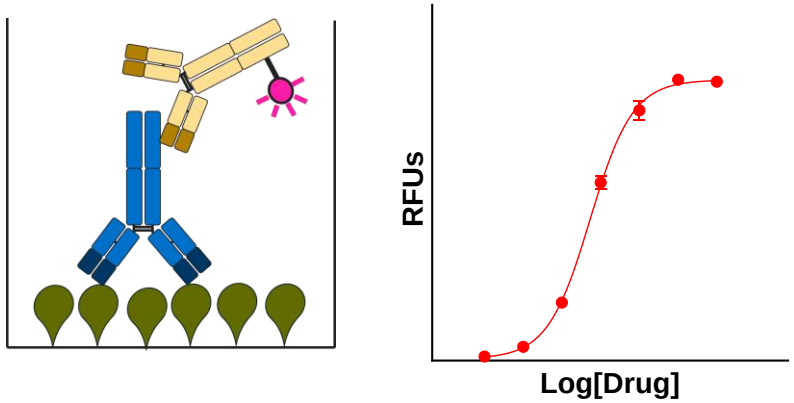


Enabling new and accelerated ADC pipelines requires outside-the-box strategies, methodologies, and technologies for CMC bioassay

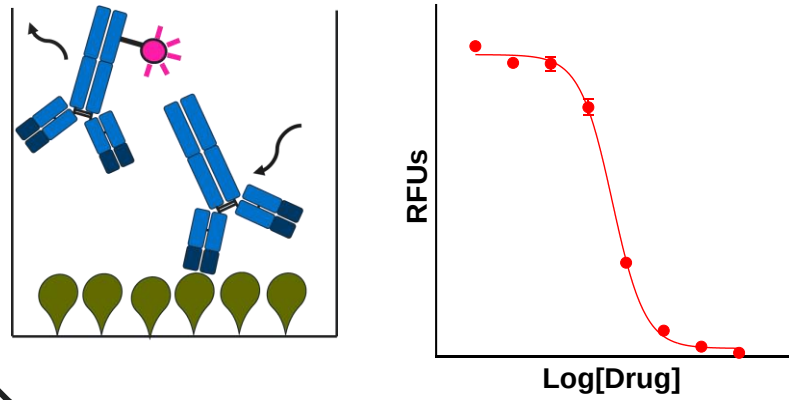
Common methods for ADC lot-release potency testing

Binding

Indirect ELISA/Immunoassay

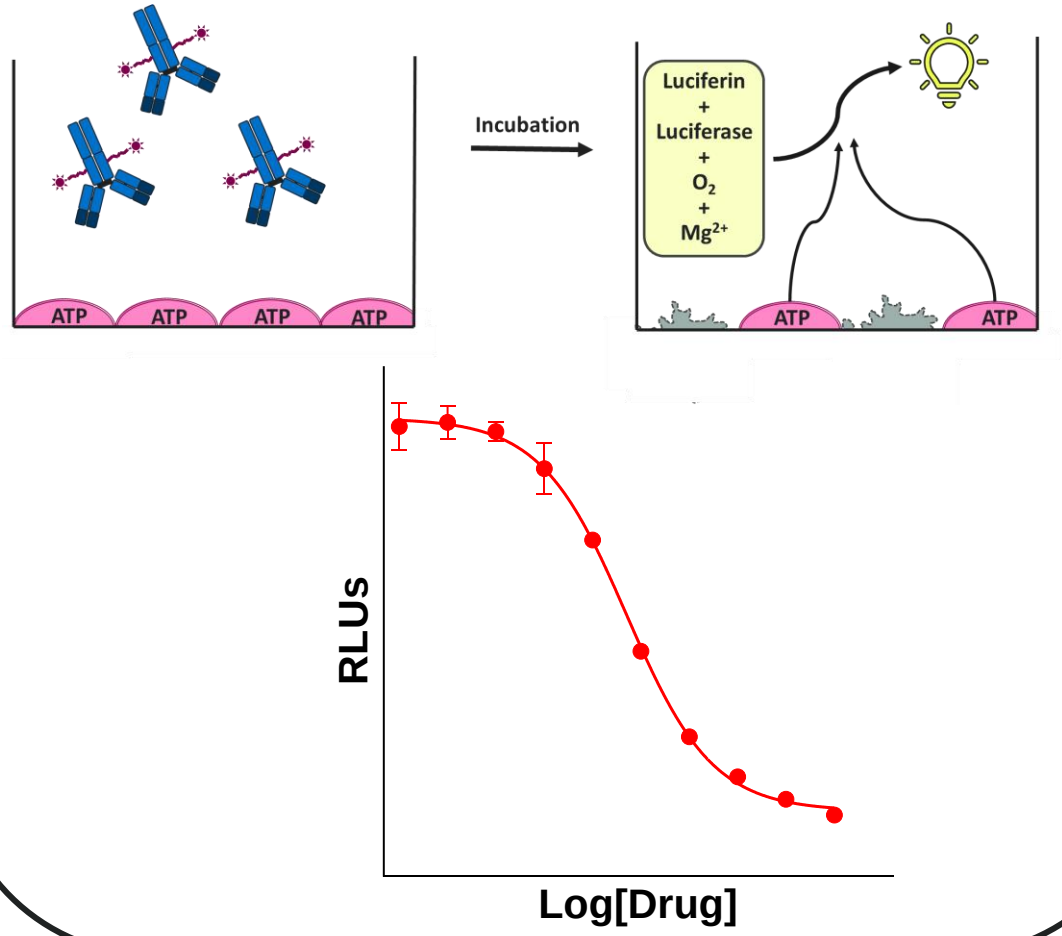


Competitive ELISA/Immunoassay

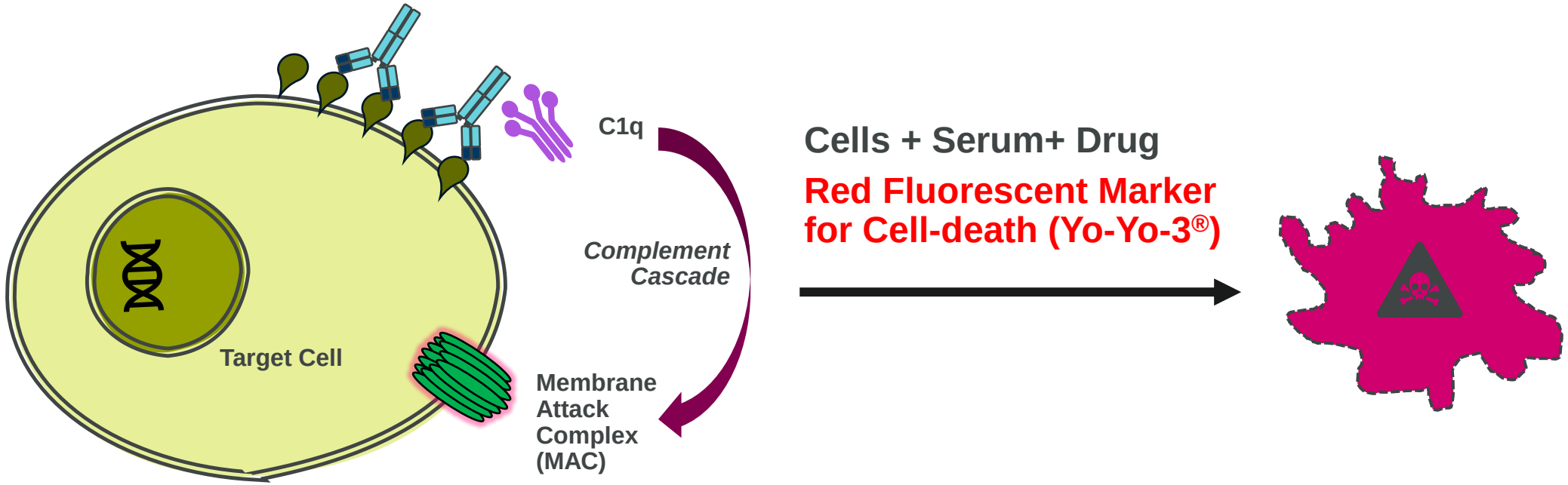


Cytotoxicity

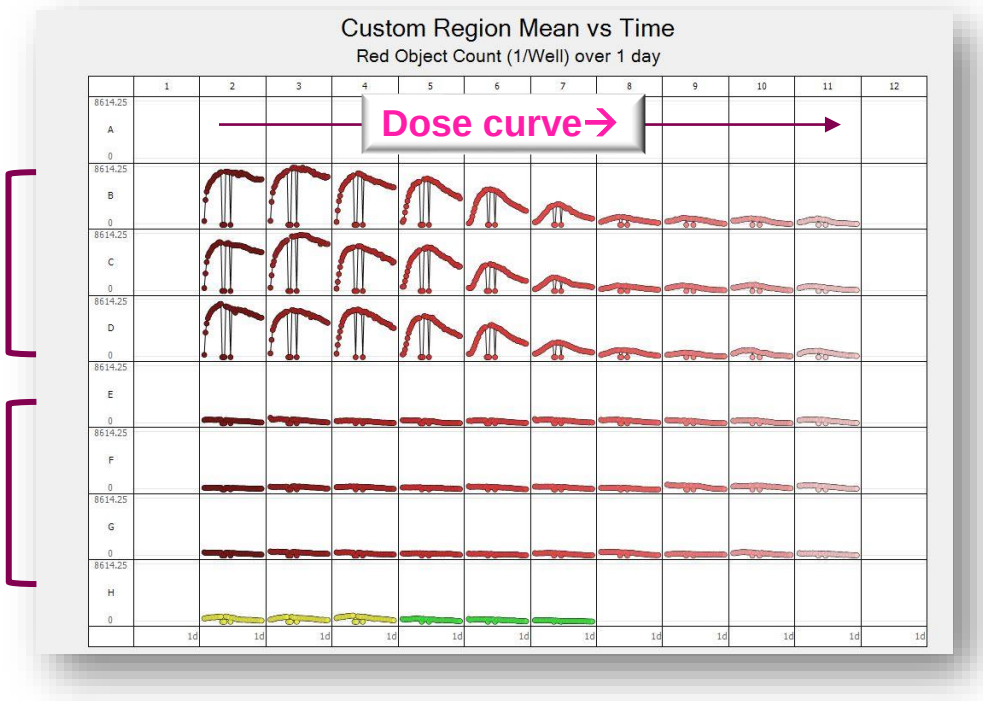
CellTiter Glo

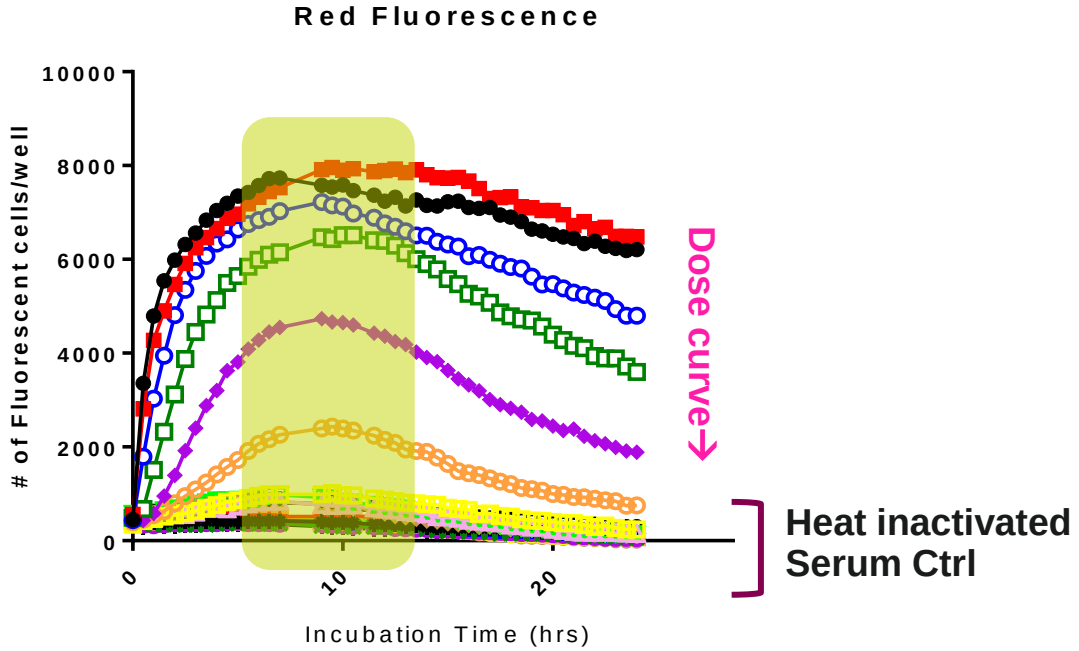


Fc Effector Function: In vitro Complement Dependent Cytototoxicity (CDC)



Using the IncuCyte to optimize CDC activity assay





- Measure fluorescence at multiple time-points within a single plate
- Read plates for days with as little as 5min intervals
- Determine assay window/incubation time