invitrogen

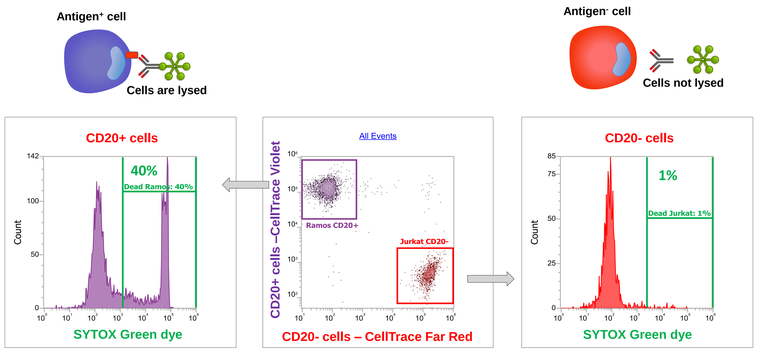
Development of in vitro immune effector function assays to better approximate the in vivo behavior of biotherapeutics and cell therapies

Chris Langsdorf, Bhaskar Mandavilli, and Yi-Zhen Hu
Thermo Fisher Scientific, Eugene, OR, USA

Background

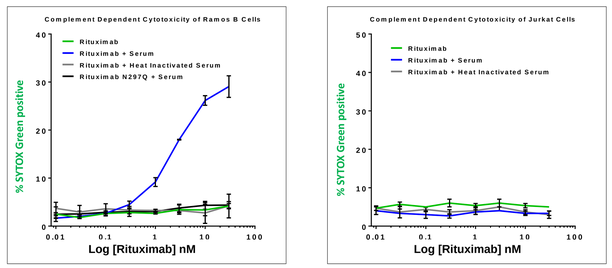
With continued growth in development and approval of biologic drugs and cell therapies, comes a need for more robust and reliable cell-based assays and analysis systems. Here we describe new methods to assess the specific killing of target cells by antibody-mediated complement-dependent cytotoxicity (CDC), antibody-dependent cellular cytotoxicity (ADCC) by natural killer cells, T cell killing, and antibody-dependent cellular phagocytosis (ADCP). These methods are demonstrated with single-cell analysis models compatible with flow cytometry and image-based analysis.

Figure 1. Specific Complement-Dependent Cytotoxicity Assay



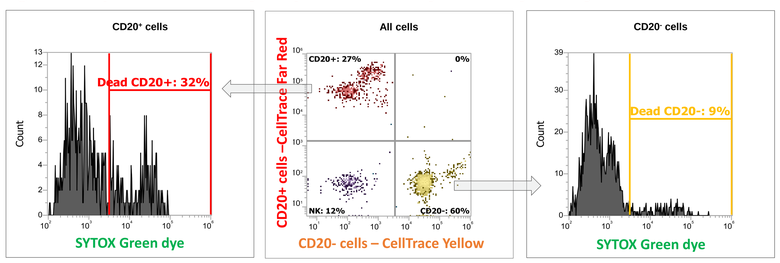
Antibody binding to antigen-positive cells triggers the classical complement pathway, leading to cell lysis. CD20-positive Ramos cells were labeled with **CellTrace Violet** Dye. CD20-negative cells with **CellTrace Far Red**. The two cell types were mixed 1:1 and incubated with 10 nM Rituximab and 10% fresh human serum for one hour. Viability measured with **SYTOX Green** on an Attune NxT Flow Cytometer with Autosampler. Cell lysis occurs in antigen-positive cells.

Figure 2. Flow Cytometry Screening for Complement-Dependent Cytotoxicity



Same methods as Figure 1. Cell lysis only occurs with functional Rituximab, active serum, and antigen-positive cells.

Figure 3. Specific Antibody-Dependent NK Cytotoxicity Assay

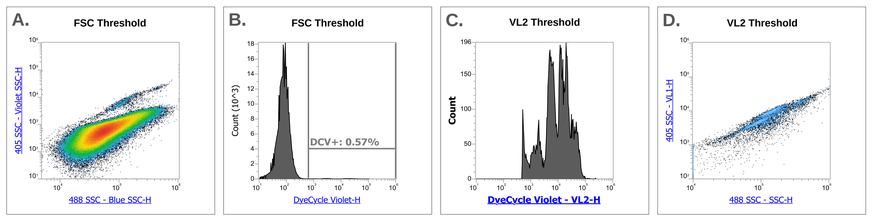


CD20+ positive: 32% dead cells

CD20 Negative: 9% dead cells

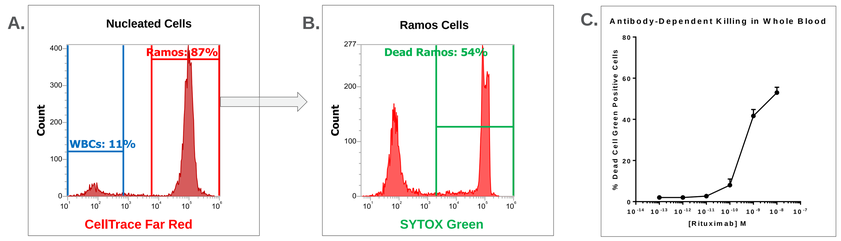
Natural killer cells were labeled with **CellTrace Violet**. CD20+ Ramos cells with **CellTrace Far Red** and CD20- Jurkat cells with **CellTrace Yellow**. Cells were mixed 1:1:1 for 1 hour at 37C, labeled with **SYTOX Green** dye and analyzed on an Attune NxT Cytometer. The three cell types appear as distinct populations on a dot plot. NK cell ADCC is observed in CD20+ cells while minimal death is observed in CD20- cells.

Figure 4. Whole Blood Analysis with the Attune NxT Flow Cytometer



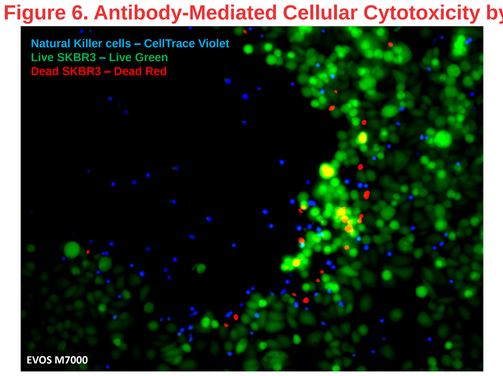
A. Hemoglobin strongly absorbs violet light, enabling label-free identification of white and red blood cells in whole blood using a dual site-scatter plot. **B.** Labeling whole blood with a vital DNA dye such as Vybrant **DyeCycle Violet** identifies nucleated blood cells. **C.** A fluorescence threshold based on DyeCycle Violet can be used to analyze only nucleated cells in whole blood. **D.** This threshold technique allows analysis of nucleated cells in whole blood.

Figure 5. Antibody-dependent Cellular Cytotoxicity in Whole Blood



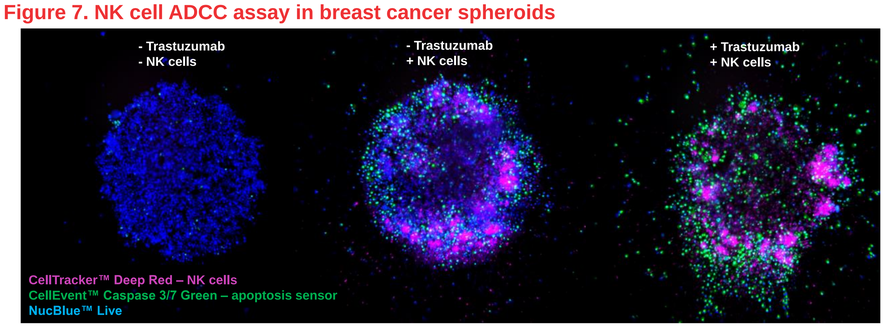
CD20+ Ramos cells were labeled with **CellTrace Far Red**. 10^5 Ramos cells were added to 5 µl human whole blood and RPMI in a final volume of 25 µl with varying concentrations of Rituximab in a deep 96-well plate. Cells were incubated 2 hours at 37C. Then 2 ml of RPMI was added to each well, along with Vybrant **DyeCycle Violet** to identify nucleated cells and **SYTOX Green** dye to identify dead cells. After 15 minutes, cells were analyzed on an Attune NxT Cytometer. **A.** Ramos cells are easily distinguished from white blood cells **B.** Treatment with 10 nM Rituximab resulted in 54% dead Ramos cells. **C.** Ramos cells in human whole blood are killed in a Rituximab-dependent manner.

Figure 6. Antibody-Mediated Cellular Cytotoxicity by Natural Killer Cells



Natural killer cells isolated from human PBMCs using DynaBeads™ Untouched NK cells were labeled with **CellTrace Violet** Dye and added to monolayer SKBR3 breast cancer cells. Cells were incubated for 2 hours with 10 nM trastuzumab and a working solution of two dyes from the LIVE/DEAD™ Cell Imaging Kit. Labeled SKBR3 cells transition from green to red upon killing by natural killer cells.

Figure 7. NK cell ADCC assay in breast cancer spheroids



Natural killer cells isolated from human PBMCs using DynaBeads™ Untouched NK cells were added to SKBR3 breast cancer spheroids with or without 10 nM trastuzumab. NK cell penetration and tumor cytotoxicity were evaluated using live-cell whole-spheroid imaging on the CellInsight CX7 LZR High Content Analysis system. Addition of NK cells induced moderate cytotoxicity, while addition of NK cells and trastuzumab resulted in substantial apoptosis and degradation of spheroid structure

Figure 8. Antibody-dependent phagocytosis model system

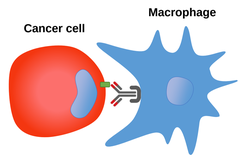
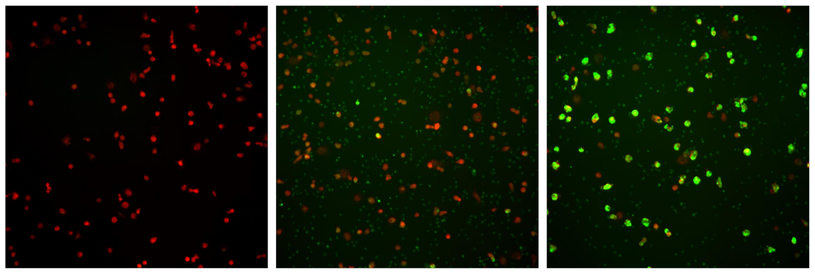
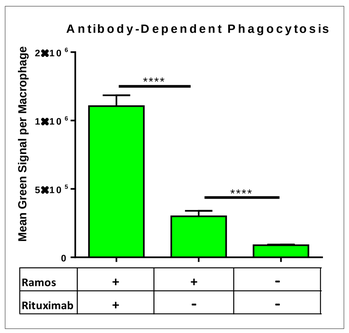


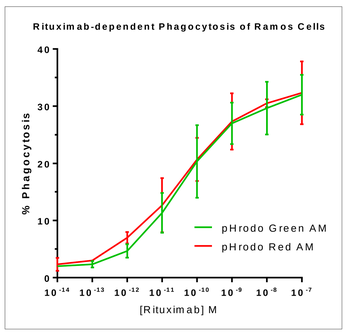
Figure 9. Direct indication of Antibody-mediated phagocytosis



Human monocyte-derived macrophages were cultured in a 96-well plate before labelling with **CellTrace Far Red**. Ramos B cells were labeled with **pHrodo Green AM Ester** and added to macrophages ± 10 nM Rituximab and incubated for four hours at 37C. Cells were imaged and analyzed with the **CellInsight CX7 LZR** high content analysis system. **Bright green fluorescence provides a direct qualitative indication of antibody-mediated phagocytic events.**

Figure 10. Quantitative Imaging of Antibody-Dependent Phagocytosis





Human monocyte-derived macrophages were cultured in a 96-well plate before labelling with **CellTrace Far Red**. Ramos B cells were labeled with **pHrodo Green AM Ester** and added to macrophages ± 10 nM Rituximab and incubated for four hours at 37C. Cells were imaged and analyzed with the CellInsight CX7 LZR high content analysis system. **Image-based analysis of single cells provides a direct quantitative indication of antibody-mediated phagocytic events.**

References

- Journal for ImmunoTherapy of Cancer 2018, 6 (Suppl 1) :114
- Journal of Immunological Methods 468 (2019) 55–60
- Methods 134–135 (2018) 149–163

Conclusions

- Flow cytometry provides a robust method to directly measure T, NK, and complement-dependent killing of cancer cells in human whole blood.
- Penetration and potency of immune effector cells can be evaluated using whole-spheroid imaging.

ThermoFisher SCIENTIFIC

invitrogen

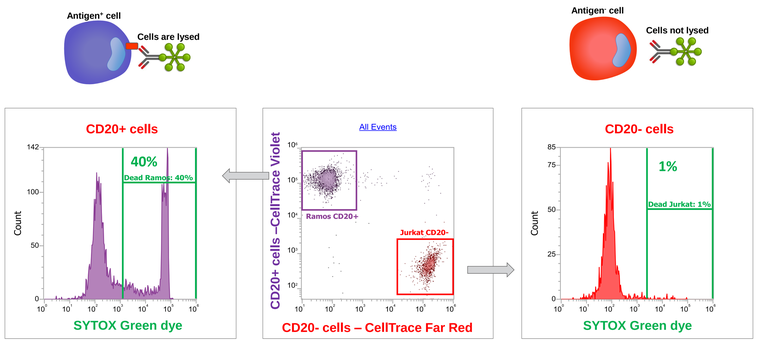
Development of in vitro immune effector function assays to better approximate the in vivo behavior of biotherapeutics and cell therapies

Chris Langsdorf, Bhaskar Mandavilli, and Yi-Zhen Hu
Thermo Fisher Scientific, Eugene, OR, USA

Background

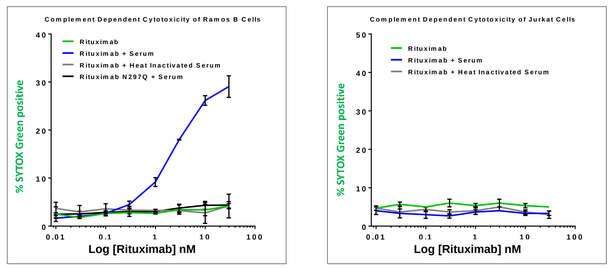
With continued growth in development and approval of biologic drugs and cell therapies, comes a need for more robust and reliable cell-based assays and analysis systems. Here we describe new methods to assess the specific killing of target cells by antibody-mediated complement-dependent cytotoxicity (CDC), antibody-dependent cellular cytotoxicity (ADCC) by natural killer cells, T cell killing, and antibody-dependent cellular phagocytosis (ADCP). These methods are demonstrated with single-cell analysis models compatible with flow cytometry and image-based analysis.

Figure 1. Specific Complement-Dependent Cytotoxicity Assay



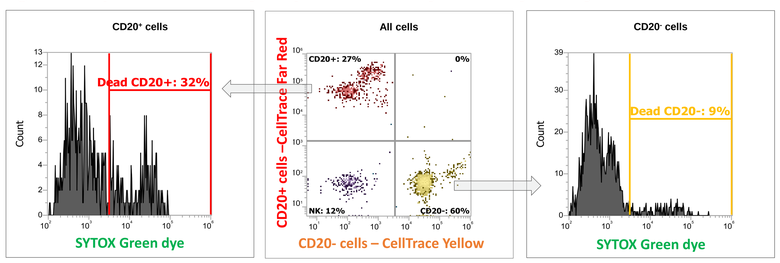
Antibody binding to antigen-positive cells triggers the classical complement pathway, leading to cell lysis. CD20-positive Ramos cells were labeled with **CellTrace Violet** Dye. CD20-negative cells with **CellTrace Far Red**. The two cell types were mixed 1:1 and incubated with 10 nM Rituximab and 10% fresh human serum for one hour. Viability measured with **SYTOX Green** on an Attune NxT Flow Cytometer with Autosampler. Cell lysis occurs in antigen-positive cells.

Figure 2. Flow Cytometry Screening for Complement-Dependent Cytotoxicity



Same methods as Figure 1. Cell lysis only occurs with functional Rituximab, active serum, and antigen-positive cells.

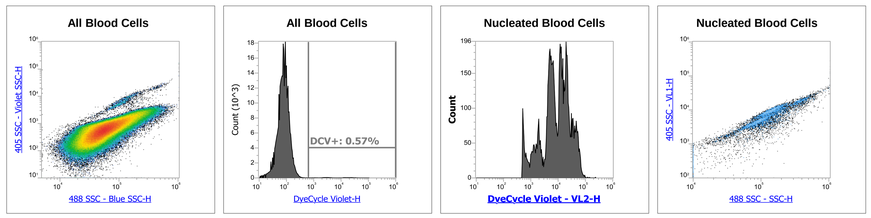
Figure 3. Specific Antibody-Dependent NK Cytotoxicity Assay



CD20+ positive: 32% dead cells **CD20 Negative: 9% dead cells**

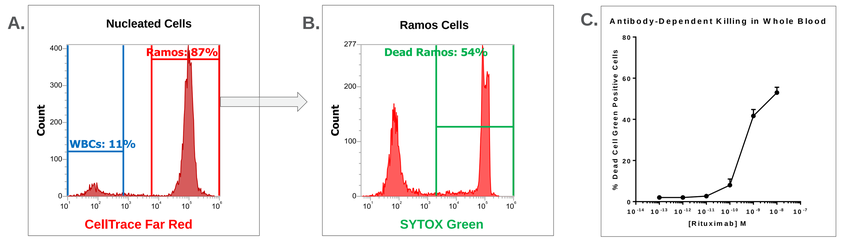
Natural killer cells were labeled with **CellTrace Violet**. CD20+ Ramos cells with **CellTrace Far Red** and CD20- Jurkat cells with **CellTrace Yellow**. Cells were mixed 1:1:1 for 1 hour at 37C, labeled with **SYTOX Green** dye and analyzed on an Attune NxT Cytometer. The three cell types appear as distinct populations on a dot plot. NK cell ADCC is observed in CD20+ cells while minimal death is observed in CD20- cells.

Figure 4. Whole Blood Analysis with the Attune NxT Flow Cytometer



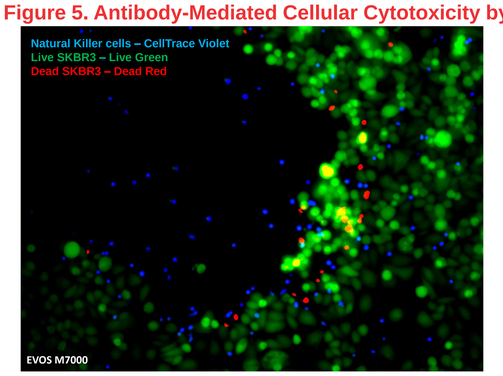
A. Hemoglobin strongly absorbs violet light, enabling label-free identification of white and red blood cells in whole blood using a dual site-scatter plot. **B**. Labeling whole blood with a vital DNA dye such as Vybrant **DyeCycle Violet** identifies nucleated blood cells. **C.** A fluorescence threshold based on DyeCycle Violet can be used to analyze only nucleated cells in whole blood. **D.**

Figure 5. Antibody-dependent Cellular Cytotoxicity in Whole Blood



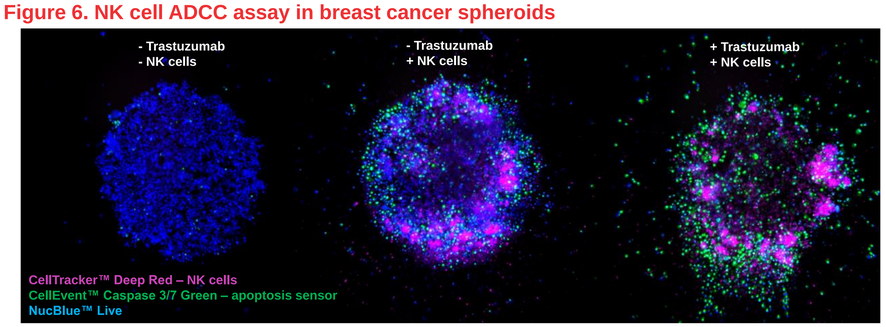
CD20+ Ramos cells were labeled with **CellTrace Far Red**. 10^5 Ramos cells were added to 5 µl human whole blood and RPMI in a final volume of 25 µl with varying concentrations of Rituximab in a deep 96-well plate. Cells were incubated 2 hours at 37C. Then 2 ml of RPMI was added to each well, along with Vybrant **DyeCycle Violet** to identify nucleated cells and **SYTOX Green** dye to identify dead cells. After 15 minutes, cells were analyzed on an Attune NxT Cytometer. **A.** Ramos cells are easily distinguished from white blood cells **B**. Treatment with 10 nM Rituximab resulted in 54% dead Ramos cells. **C.** Ramos cells in human whole blood are killed in a Rituximab-dependent manner.

Figure 5. Antibody-Mediated Cellular Cytotoxicity by Natural Killer Cells



Natural killer cells isolated from human PBMCs using DynaBeads™ Untouched NK cells were labeled with **CellTrace Violet** Dye and added to monolayer SKBR3 breast cancer cells. Cells were incubated for 2 hours with 10 nM trastuzumab and a working solution of two dyes from the LIVE/DEAD™ Cell Imaging Kit. Labeled SKBR3 cells transition from green to red upon killing by natural killer cells.

Figure 6. NK cell ADCC assay in breast cancer spheroids



Natural killer cells isolated from human PBMCs using DynaBeads™ Untouched NK cells were added to SKBR3 breast cancer spheroids with or without 10 nM trastuzumab. NK cell penetration and tumor cytotoxicity were evaluated using live-cell whole-spheroid imaging on the CellInsight CX7 LZR High Content Analysis system. Addition of NK cells induced moderate cytotoxicity, while addition of NK cells and trastuzumab resulted in substantial apoptosis and degradation of spheroid structure

Figure 7. Antibody-dependent phagocytosis model system

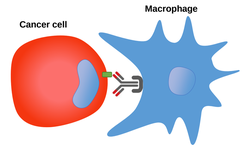
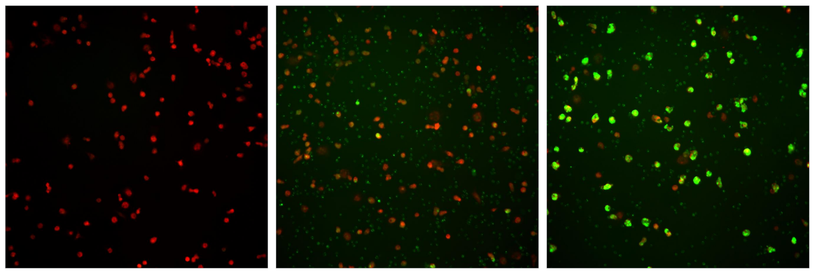
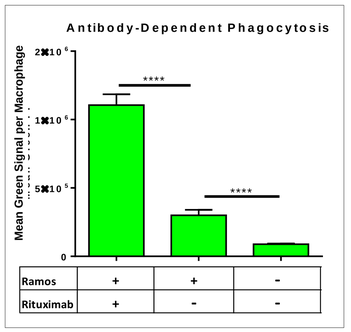


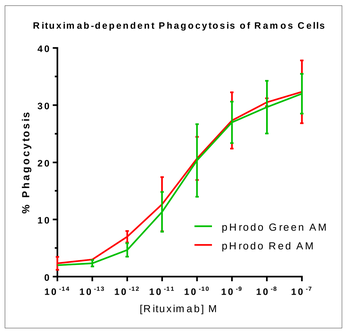
Figure 8. Direct indication of Antibody-mediated phagocytosis



Human monocyte-derived macrophages were cultured in a 96-well plate before labelling with **CellTrace Far Red**. Ramos B cells were labeled with **pHrodo Green AM Ester** and added to macrophages ± 10 nM Rituximab and incubated for four hours at 37C. Cells were imaged and analyzed with the **CellInsight CX7 LZR** high content analysis system. **Bright green fluorescence provides a direct qualitative indication of antibody-mediated phagocytic events.**

Figure 9. Quantitative Imaging of Antibody-Dependent Phagocytosis





Human monocyte-derived macrophages were cultured in a 96-well plate before labelling with **CellTrace Far Red**. Ramos B cells were labeled with **pHrodo Green AM Ester** and added to macrophages ± 10 nM Rituximab and incubated for four hours at 37C. Cells were imaged and analyzed with the CellInsight CX7 LZR high content analysis system. **Image-based analysis of single cells provides a direct quantitative indication of antibody-mediated phagocytic events.**

References

- Journal for ImmunoTherapy of Cancer 2018, 6 (Suppl 1) :114
- Journal of Immunological Methods 468 (2019) 55–60
- Methods 134–135 (2018) 149–163

Conclusions

- Flow cytometry provides a robust method to directly measure T, NK, and complement-dependent killing of cancer cells in human whole blood.
- Penetration and potency of immune effector cells can be evaluated using whole-spheroid imaging.

ThermoFisher SCIENTIFIC