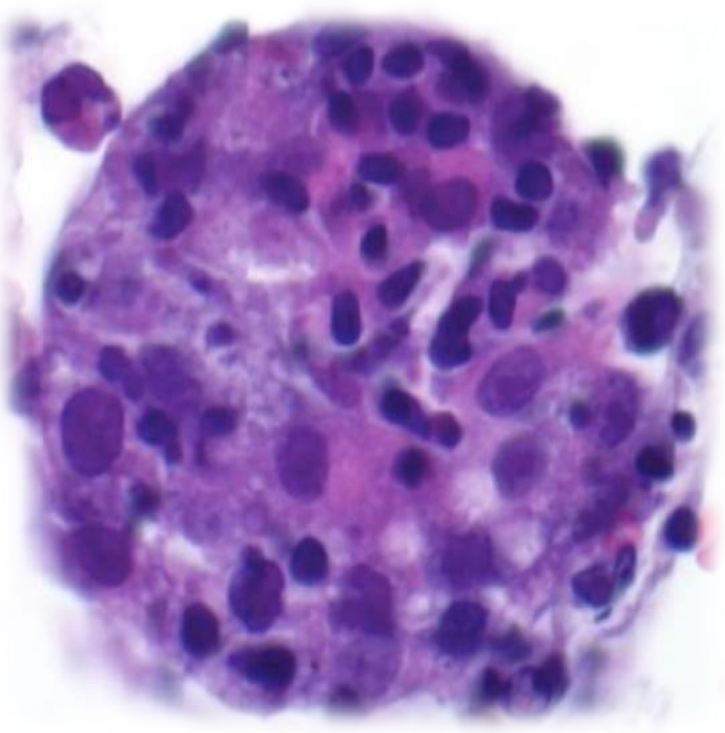
APPLICATION NOTE

STING: rational drug combinations development

This application note outlines how STING agonists in rational combination with checkpoint inhibitors enhance response to treatment.



What are tumoroids?

Up to 9,000 tumoroids are created from a patient's tumor. Each tumoroid is spherical and ~150µm in diameter - a true micro-version of the Tumor MicroEnvironment (TME). Unlike conventional tumor organoids, there are no chemical dissociation processes, no propagation steps using growth factors, no attempt to reassemble the complex mixture of constituents that are the tumor microenvironment.

The immune compartment

A completely unique approach to enable the detailed analysis of drug effects on multiple cell types in the tumor microenvironment and tumor-immune cell interactions.
Nilogen has combined its powerful tumoroid platform with unique high-content confocal microscopy assays for tumor cell killing, creating a profoundly new approach to rational drug combination development.

ADDITIONAL CONTENT

Tumoroids - the difference compared to tumor organoids?

LEARN MORE

The Innate Immune System

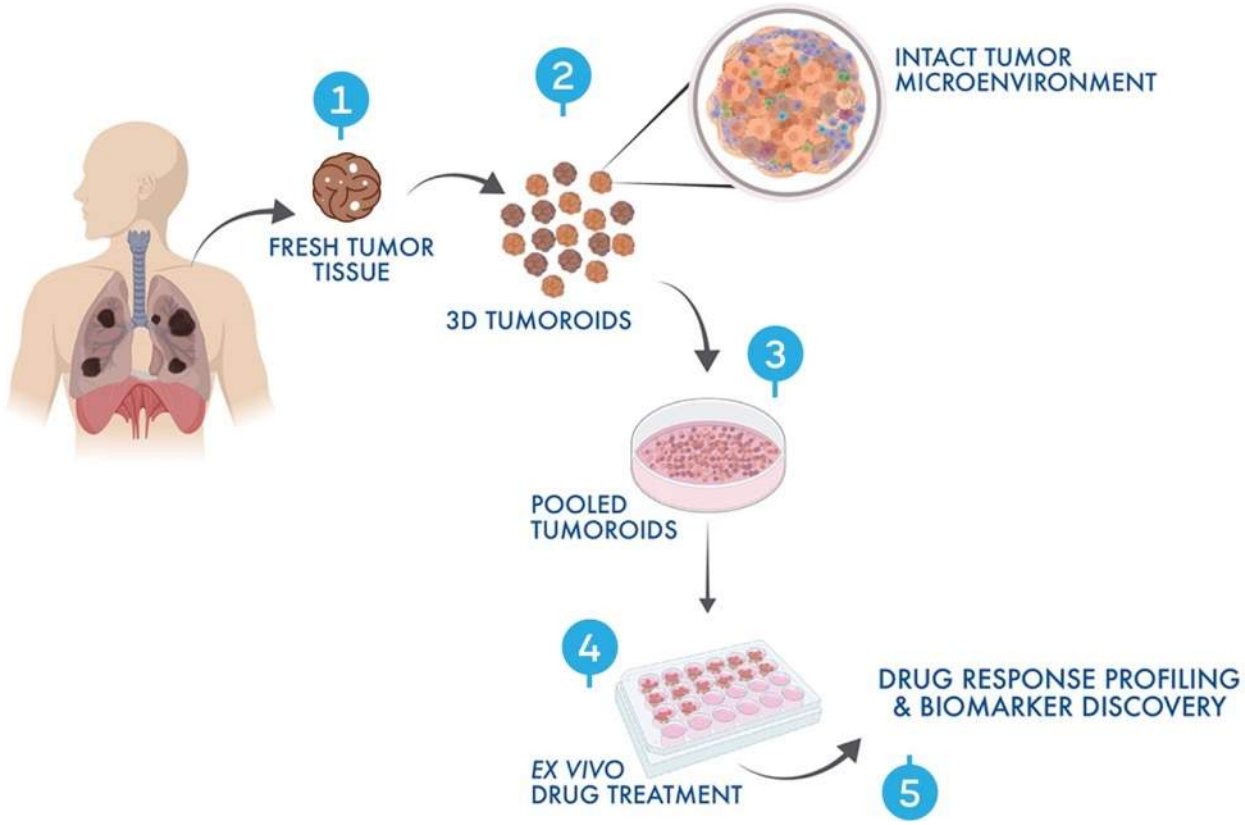
The continued development of the field of immune-oncology has informed our understanding of the complexity of the tumor microenvironment and tumor-immune cell interactions. While immunotherapies have become the standard of care for many cancers, there is still a significant unmet need as the majority of patients either are not candidates for immunotherapy or do not respond. Combination therapies are being investigated to widen the population of patients who could benefit from immunotherapy and improve response rates. This has driven the emerging interest in ways to stimulate the innate immune system, in particular efforts to modulate Toll-Like Receptors (TLRs) and the STING (stimulator of interferon genes) pathway to drive antigen-presenting cell activation and tumor antigen presentation to effector T cells. STING plays an important role in the innate immune system, and when STING is activated, inflammatory cytokines and interferons are produced. Those inflammatory proteins then activate the innate immune system, driving the production of T cells. Activation of the STING pathway has therefore been seen as an opportunity to boost the body's T cell response. STING programs combined with existing checkpoint inhibitor programs are being explored as a way to elicit an increased response in patients to immunotherapeutic interventions. Reducing innate immune barriers to anti-tumor immunity, to promote robust, localized response in the tumor microenvironment (TME) and address resistance to T-cell-based immunotherapies are active avenues for investigation. The first generation of STING agonists needed to be directly injected into tumors, which limited their application to a small number of cancer types. These initial molecules were designed as analogs of cyclic dinucleotide (CDN), which STING binds to, but may lead to activation of STING in "off- target" cell types, such as effector T cells, and can induce inflammatory cytokines when administered into the circulatory system, resulting in apoptosis and ultimately impeding the formation of immunological memory. Alternative approaches targeting STING agonists to intratumoral Antigen Presenting Cells (APCs) could reduce these non-targeted, systemic effects and improve the overall efficacy of such approaches. Today, there is renewed interest are pursuing 2nd generation non-CDN-based STING agonists which may be suitable for systemic delivery, and localized modulation through the use of bacterial vectors for targeting STING agonism to APCs, which are actively phagocytosed and trigger complementary immune pathways.
We set out to investigate whether STING agonists could enhance checkpoint inhibition to expand patient response.

A uniquely holistic approach

Studying all the potential options and combinations requires a model that contains the complete human tumor microenvironment and can directly compare and contrast multiple therapeutic interventions in the same tumor, equivalently. Human tumors are incredibly complex and regional variations mean that heterogeneity is a very significant issue to resolve. Additionally, human tumors may develop over several years, even decades, and the continual cell death, renewal, wound healing process and vasculature creates stroma which may be highly desmoplastic and resistant to penetration by therapies. Mouse models which grow in days or weeks cannot recapitulate the true tumor microenvironment, and organoids are only a pale reflection of the complexity found in real tumors. Tissue slices or histoculture suffer from lacking representation of the complete tumor, and even with many replicates, variance between study arms is significant. Traditional models have been a significant factor in the lack of successful translation of molecules into the clinic. Failure rates remain stubbornly high at over 90%, with at least 50% of these being due to lack of efficacy in patients. So what might be an option prior to going straight to a first in human study to avoid the expense and patient suffering?

Over several decades, Dr Soner Altiok, now co-founder and CSO of Nilogen Oncosystems, and a board-certified pathologist, through his relationships with clinical research teams had utilized all the classical models systems in an effort to improve drug development and improve patient care. He realized there was a need for a different approach. One that could capture the complexity of a human tumor, remain stable for long enough to provide reproducible assay results, and somehow overcome the issue of heterogeneity found in every tumor. Dr Altiok's eureka moment was to develop a mechanical process which could process a human tumor in minutes into thousands of mini-tumors, "tumoroids", all of equivalent size and shape, without the use of enzymatic digestion, and not requiring reassembly or propagation. Each tumoroid is variable in its composition, but contains a representation of the TME, ECM and complement of both immune and tumor cells found in the original patient tumor tissue (**Figure 1**). The tumoroids were pooled to create a homogeneous mixture and an equal number aliquoted into each assay well, typically around 400. Using this approach, each well containing tumoroids from the same patient tissue was highly repeatable, with very low well-to-well variability (**Figure 2**).

Figure1: The process of creating tumoroids



1. ISOLATION OF FRESH TUMOR TISSUE

We start with freshly isolated tissue ethically sourced from our extensive network of clinics in the US.

2. CONVERSION INTO 3D TUMOROIDS

Each fresh tumor is minimally processed using a proprietary mechanical method into uniformly-sized tumoroids of about 150 µm in diameter. No chemical dissociation, propagation or reassembly.

3. POOLING AND ALIQUOTING OF TUMOROIDS

To account for the heterogeneity that exists within each tumor, we pool up to 9,000 tumoroids from a single tumor before aliquoting hundreds of tumoroids into individual wells of a multi-well plate, minimising well-to-well variability.

4. EX VIVO TREATMENT WITH CANDIDATE THERAPIES

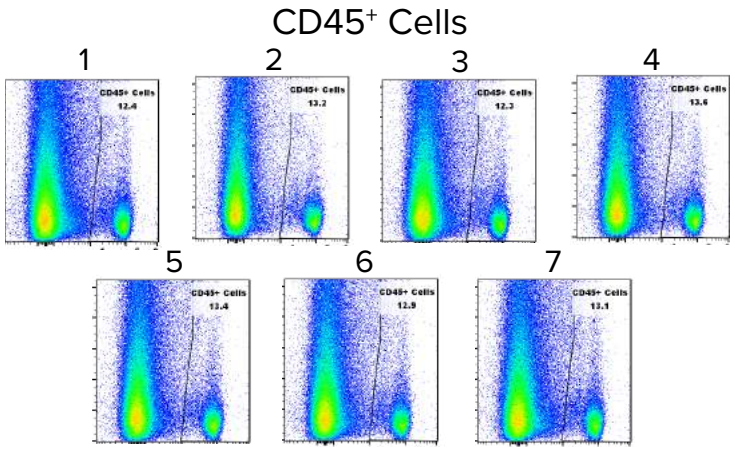
A single tumor can provide enough tumoroids for many study arms with multiple assays per arm, which are then exposed to drug/combinations. Each drug/combination can be assessed using multiple analytical techniques, resulting in a comprehensive, multi-omics view of multiple drugs/combinations in each tumor.

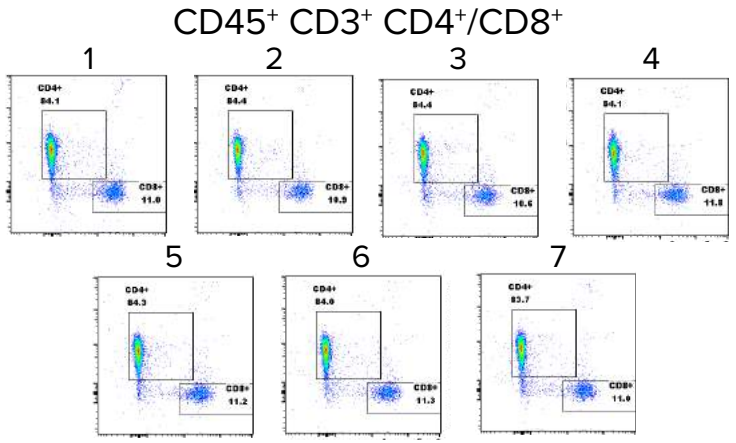
5. DRUG RESPONSE PROFILING & BIOMARKER DISCOVERY

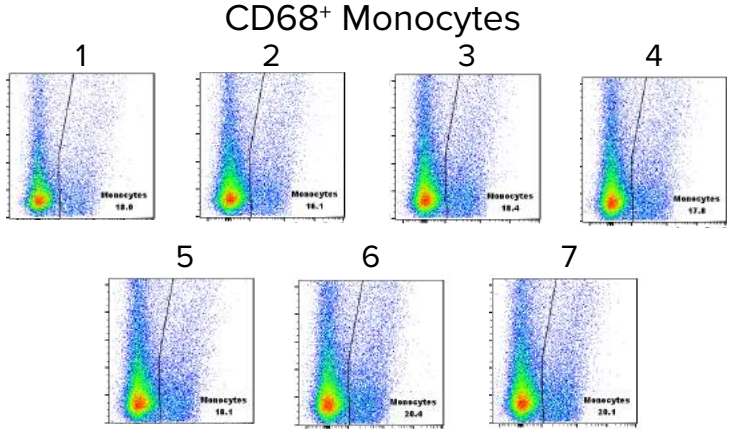
Nilogen uses sophisticated bioinformatics and artificial intelligence algorithms to analyze and integrate your data, ultimately delivering a fully analyzed, interpreted, and actionable report of your study.

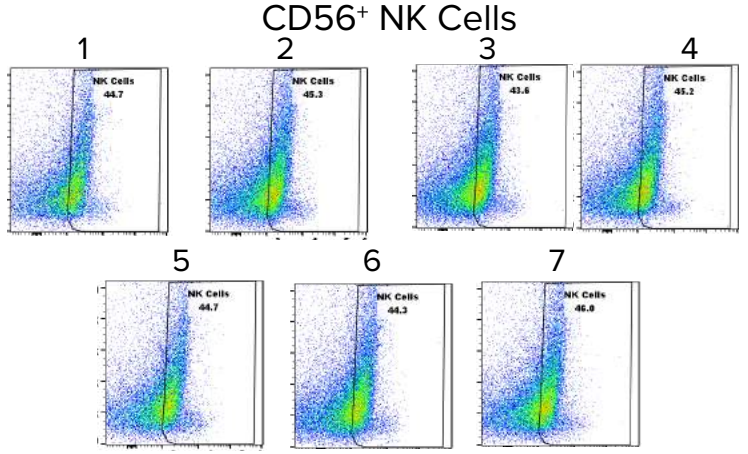
Figure2: Minimal well to well variability

7 wells of tumoroids, created from a pool of tumoroids from a single patient tumor tissue. Flow Cytometry data from each well show results for multiple cell types. Table on RHS shows minimal variation from well-to-well.









Pooled tumoroids were evenly distributed in seven wells

Cell Type	Mean Percent	Variance
CD45⁺	13.0%	0.2
CD68⁺ Monocytes	18.4%	1.8
CD45⁺ CD3⁺ CD4⁺/CD8⁺	84.1% / 11.1%	0.1 / 0.1
CD56⁺ NK Cells	44.8%	0.5

This approach therefore overcame the issue of tumor heterogeneity and enabled many assays to be run side by side, so that a significant number of therapies and combinations could be tested in the same patient tissue – something simply not possible in other models or a patient.
Over several years, Nilogen has taken the tumoroid technology and developed a comprehensive and unique suite of assays that are able to evaluate not just efficacy, but quantify penetration, phagocytosis, and ultimately tumor cell killing, as well as many phenotypic and functional assays to understand drug mechanism of action, activity and develop effective biomarker strategies. This one platform, 3D-EXplore (**Figure 3**), enables the evaluation and direct comparison of many drug modalities from conventional chemotherapeutics and small molecule therapies to monoclonal antibodies, bi and tri-specifics, Antibody Drug Conjugates, Antibody Dependant Cellular Cytotoxicity, Adoptive Cell Therapies and now oncolytic viruses.

In 2020 at AACR, Nilogen published a poster about the development of rational drug combinations using 3D-EXplore, specifically exploring the combinations of cGAS-STING agonists ADU-S100 and 2'3'-cGAMP with Nivolumab. This led to some exciting discoveries demonstrating the capabilities of 3D-EXplore to directly compare multiple combination therapies in the same fresh patient tissue using both conventional and assays unique to Nilogen. Multiple study arms comparing Control, ADU-S100, 2'3'-cGAMP, Nivolumab, Nivolumab+ADU-S100 and Nivolumab+2'3'-cGAMP were created and in **Figure 4** the Flow Cytometric analysis of CD45 populations shows an equal distribution of processed tumor tissue across each treated well. However, analysis of the immune populations showed that treatment with Nivolumab alone or in combination with cGAS-STING agonists, consistently displayed increased expression of several activation markers in both T-cell subsets and granulocytes.
Supernatants from the Flow Cytometry wells were collected at 48 hours for multiplexed cytokine analysis. **Figure 5** shows the results for several cytokines where there was a strong induction in those wells treated with combinations of STING agonist and checkpoint inhibitor, including TNF, and IL10, which play a major role related inducing a strong antigenic response in dendritic cells. Dendritic cells connect the innate and adaptive part of the immune system and play a pivotal role in cancer.
Ultimately tumor cell death is a key measure in evaluation of a therapeutic intervention. Nilogen's unique high-content confocal

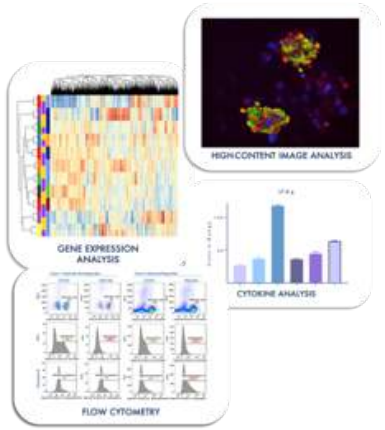
Figure 3: Nilogen's 3D-EXplore platform provides a holistic approach to drug development, enabling a deep understanding and comparison of the mechanism of action of multiple drug therapies in the same patient's tumor.

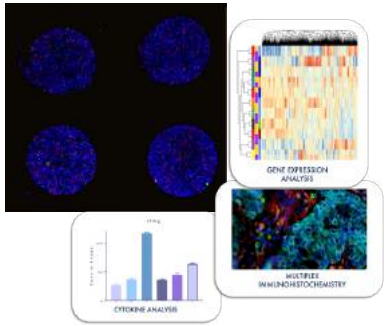
Utilizing leading-edge technologies:

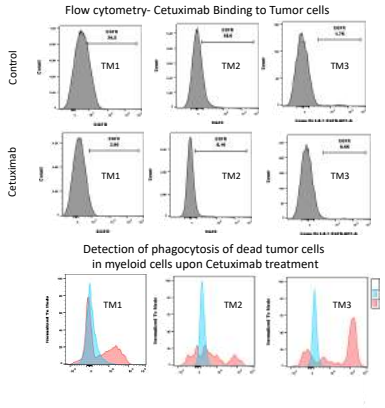
- High content imaging
- Multiplex IF
- Flow cytometry
- NGS/WGS
- RNAseq/Nanostring
- scRNAseq
- CITE-seq
- Cytokines
- Phagocytic activity
- Tumor Cell Killing
- RNA/FFPE prep

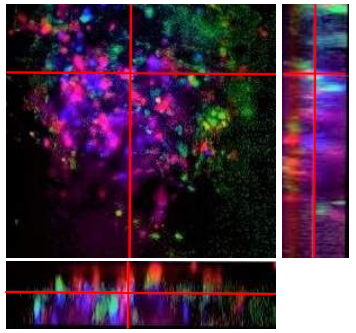
Therapeutic Modalities Validated:

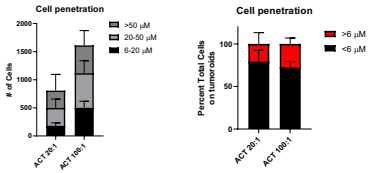
- I-O mAbs/multispecifics
- ACT
- ADCs
- ADCCs
- Oncolytic Viruses
- Small molecules
- Chemotherapies

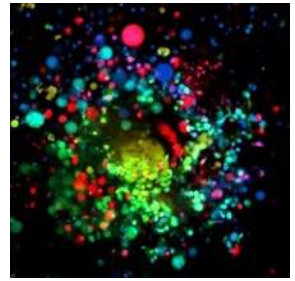












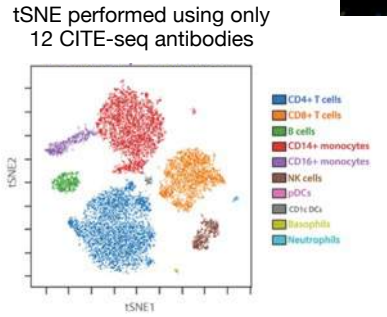
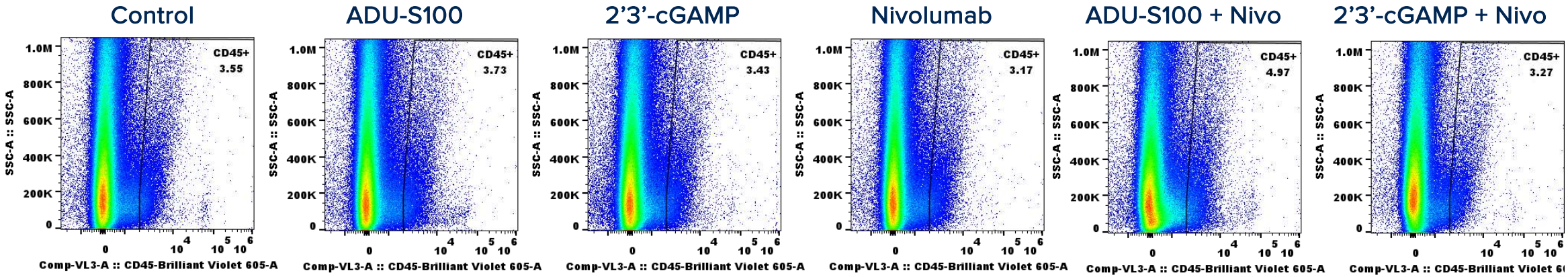


Figure 4: Flow Cytometry analysis of CD45 populations from treatment arms



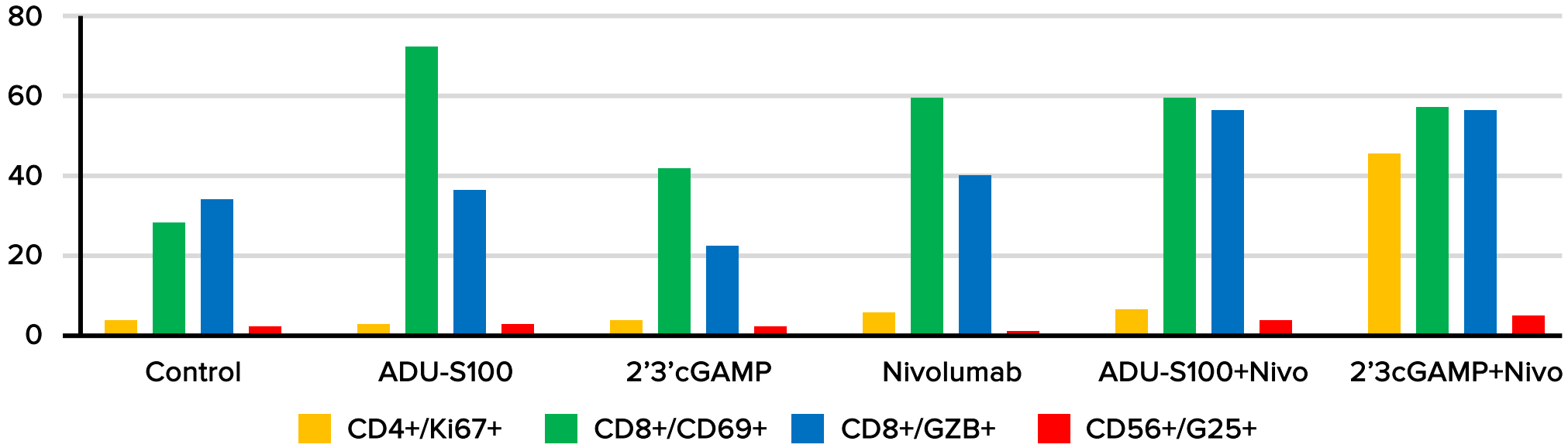
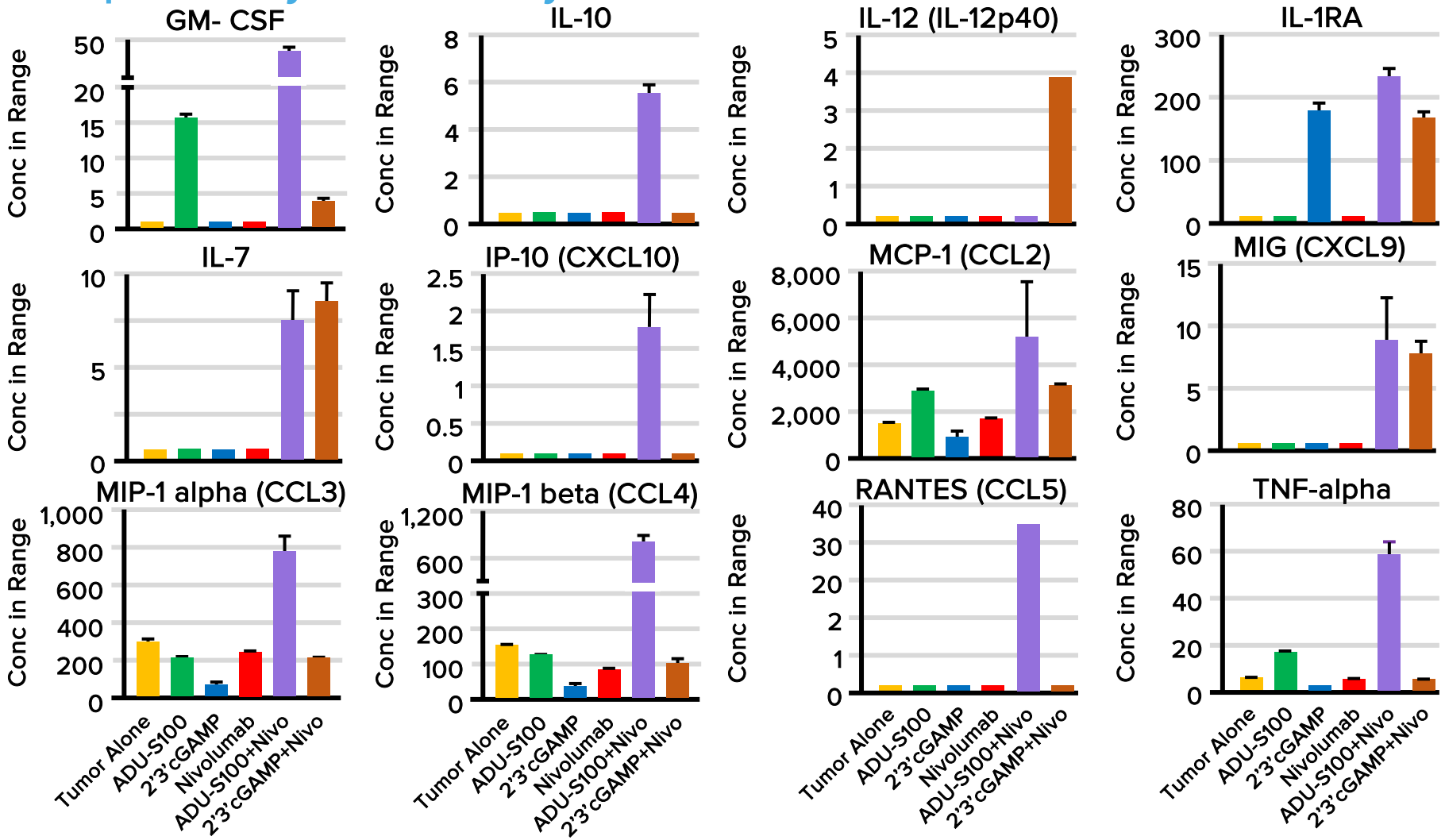


Figure 5: Multiplexed cytokine analysis of treatment arms



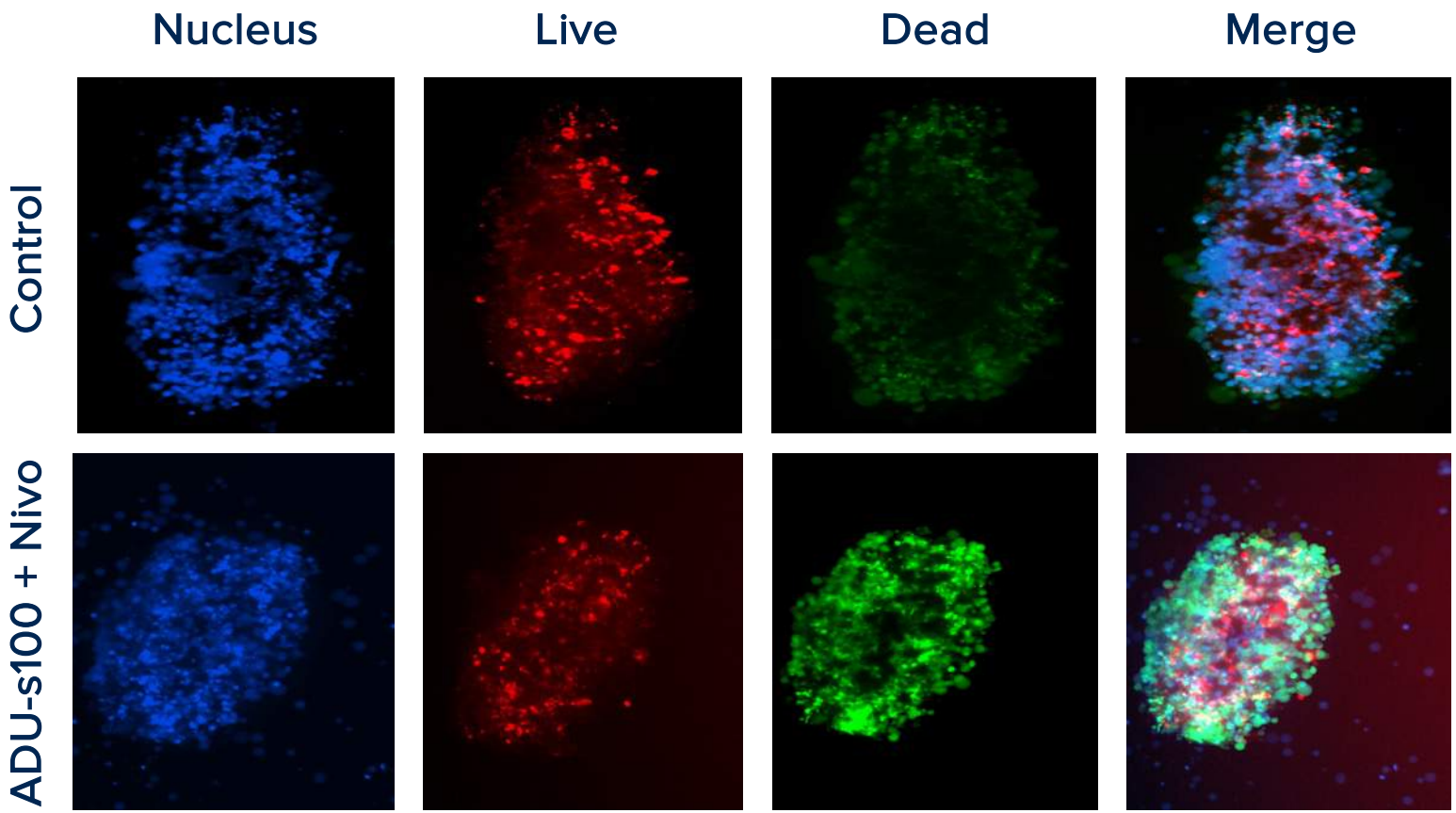
microscopy assay enables the direct comparison of treatments to control (untreated) tumoroids.
A proprietary stain to detect live cells in the tumoroids is incorporated at the beginning of the assay. At the end of the assay, a stain to detect cell death is then added. Cells that stain for both the live and dead labels are then measured - these cells were killed during the course of the assay. The treatment arms are then normalized against the untreated control (baseline) to determine cell death during the assay (**Figure 6**).

Results

The results of the study showed that STING pathway agonists lead to activation of tumor-resident T-cells in colorectal carcinoma, and that Nivolumab enhanced immune-modulatory effects of STING activators, suggesting a potential interaction between these therapeutic agents, and that

Figure 6: Tumor Cell Killing Assay

Tumor Cell Killing assay was performed using high content confocal imaging to visualize treatment-mediated changes in viability of tumor cells within the live tumoroids. Images show increased tumor cell death with the combination of ADU-S100 + Nivolumab as compared to controls.



the combination of STING agonists with Nivolumab may have clinical benefit in colorectal cancer treatment.
High content confocal imaging of the 3D tumoroid microenvironment enabled the detection of enhanced tumor cell killing in response to varying treatment conditions. This analysis demonstrated enhanced cytokine release and increased tumor cell death in the 3D tumor organoids, compared to controls. There was a strong induction of many cytokines where tumoroids are treated with combinations of STING agonist and checkpoint inhibitor, including TNF, and IL10, which play a major role in inducing antigenic response.

References

Ehrhart JC, Pabon MM, Pastoor T, Kreahling J, Altiok S. Abstract 6652: **Employing *ex vivo* 3D-Screen℠ technology for the development of rational combinations of immune-oncology drugs in tumor organoids of fresh patient tissue.** Proceedings of the American Association for Cancer Research Annual Meeting 2020; April 27 - 28 and June 22 - 24, 2020. *Cancer Research*. 2020; 80:16 Suppl. DOI: 10.1158/1538-7445.AM2020-6652

"The combination of immune checkpoint inhibitors and STING agonists consistently displayed increased expression of several activation markers in both T-cell subsets and granulocytes."

At Nilogen, our vision is to improve the treatment and quality of life for cancer patients by helping companies developing the oncology therapies of the future, improving their chance of succeeding in clinical trials through the application of game-changing human tumor tissue assays which provide a comprehensive analysis of drug impact on the immune compartment as well as tumor cells.

What will you discover today?

NILOGEN ONCOSYSTEMS

SOLUTIONS THAT POWER TRANSLATIONAL ONCOLOGY

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