Immuno-oncology research

A paradigm shift in supporting research into therapeutic approaches to combat cancer
Recent breakthroughs in immuno-oncology research have translated into a different way of thinking with regards to cancer. Immuno-oncology therapy is believed by many to be the most significant paradigm shift in the treatment of metastatic cancers in decades. This therapy has resulted in the ability to cause long-lasting tumor regression where surgery, radiotherapy, chemotherapy, and targeted therapy have proven less effective [1,2,4].

Thermo Fisher Scientific offers many research platforms and products to help better understand the systems and details of immuno-oncology to better understand its power to combat cancer.

**T cell immuno-oncology therapy**

Immuno-oncology therapies use genetically modified T cells to attack and kill the cancer cells. T cells do not normally identify cancer cells as foreign that would mark them for destruction. For immunotherapy to work, T cells are genetically manipulated to enable them to identify the cancer cells as an enemy and kill them as they would other foreign bodies. The more popular methods of immuno-oncology therapies can be divided into two approaches. The first is the cytotoxic T lymphocyte. This method is able to target both intracellular and cell surface antigens. The other, more discussed method, are chimeric antigen receptor (CAR) T cells that rely on cell surface expressing antigens. Both methods are described in more detail below.

**Cytotoxic T lymphocyte therapy**

Cytotoxic T lymphocyte therapy incorporates the use of genetically modified T cell receptors (TCRs) of cytotoxic T cell lymphocytes to recognize tumor antigen in the context of human leukocyte antigens (HLAs). It is this ability to recognize both cell surface and intracellular proteins that provides this method a broader array of tumor-associated targets. This is a very important point of differentiation to note as many of the other immuno-oncology methods are unable to be directed against intracellular targets [1]. The lack of tumor-exclusive antigens makes this method very difficult.

**Chimeric antigen receptor (CAR) T cell therapy**

CAR T cell therapy is another immuno-oncology method that is being heavily studied currently. These T cells are genetically modified with CARs containing antibody-based recognition domains (scFv) against cell-surface antigens linked to intracellular signaling sequences to overcome their tumor’s tolerance [4]. This allows CAR T cells to overcome the constraints of MHC-restricted T cell receptor recognition and avoid tumor escape through impairments in antigen presentation or HLA expression. The ability to modify T cell signaling moieties in the CAR T cell enables a broader functional effect than endogenous TCRs (Figure 1).

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Figure 1. Figurative illustration of two popular methods, CAR T cells (left) and cytotoxic T lymphocyte therapy (right), currently in development for immuno-oncology treatment.
The question still remains if CAR T cell therapy can be used to effectively treat a wide array of solid tumor types. Currently, approximately 50% of CAR T cell trials focus on hematological malignancies primarily targeting the B cell marker CD19 for targeting B cell leukemias and lymphomas [1]. CD19 is an excellent therapeutic target as it is present only on normal B cells, but is not present on normal tissues and is not shed into the circulating blood. Solid tumors are more difficult to treat than blood cancers for many reasons. There are many proteins on the cell surface of solid cancer cells that are also found on normal cells. Attacking such targets therefore, could result in complete destruction of certain organs [1].

Researchers are searching for the next “CD19” that would allow them to use CAR T cells with solid tumors. Recent advances in traditional cell analysis platforms such as flow cytometry have enabled for the high-throughput, multi-parametric screening of single cells. This has given rise to a greater ability to discover new potential targets for CAR T cell therapy and test their efficacy.

T cells are isolated and genetically modified to create the CAR T cells, directed to the target of interest with the appropriate intracellular signaling portion of the receptor. The difficulty lies in choosing the appropriate T cell subsets for expansion and modification [1]. In addition to the availability of T cells, the presence of regulatory T cells (Tregs) must be minimized as high levels of Tregs are predicted to potentially block the antitumor response of CAR T cells [1,4]. The engineering of the CAR T cells for resistance to immunosuppression could be beneficial to the therapeutic outcome [1]. Other challenges include dense organ tumors saturating their surroundings with signaling molecules, such as PD-L1 that turn off T cells. This defense process is the one that checkpoint inhibitors interfere with. It is not unlikely that CAR T cell therapy could one day be a combination of T cell subsets that are modified to create the most conducive environment for the anti-tumor effects.

**CAR T cell genetic engineering**

Current CAR T cell therapy utilizes what is referred to as third generation CAR T cells. The first generation CAR T cell therapy was a safe approach, but not very effective. The second and third generation CAR T cells improved on the original model by including a costimulatory receptor signaling domain from members of the TNFα superfamily and included such domains as CD28, 4-1BB (CD137), or OX40 (CD134) in the cytoplasmic portion of the CAR [1,4]. These CAR T cells exhibited greater increases in activation, proliferation, survival, cytokine secretion, anti-tumor cytolytic activity, and reactivation upon secondary stimulation [1,4].

The activities of these newly created CAR T cell models can be determined using both the cellular and biomarker profiling. The levels of CAR T cells in the subject can easily and readily be monitored though flow cytometry while the quantitative cytokine profile is measured through the use of multiplex immunoassays. With these tools, researchers can continue to improve the CAR T cells to get the right amount of positive anti-cancer effects, while reducing severe adverse events.

**Toxicity and severe adverse events (SAE)**

Immu-no-oncology therapy is sometimes thought of as having limited side effects. While this is true in relation to traditional chemotherapy, this does not mean that side effects do not exist. The types and intensity of the SAEs are dependent on a number of factors that include the design of the CAR T cells and the targeted molecule(s) of the CAR T cell [1]. The most common adverse off-target side effect is cytokine release syndrome (CRS). This is an event when a storm of cytokines are released in the patient creating a cascade of very negative effects. It is difficult to determine the origins of the cytokines and the amount that can be directly attributed to the CAR T cell. Other cell types such as the leukemia cells and macrophages brought to this area as part of the immune response release a great deal of cytokines [1]. Furthermore, it is still not completely clear if treatments aimed at containing this cytokine storm in any way negatively affect the antitumor effect of the CAR T cells. Tools such as multiplex immunoassays, which allow for the simultaneous profiling of these cytokines in a variety of sample types, give researchers a better understanding of the systems at play.

**References:**

Flow cytometry

Flow cytometry is a valuable tool used across the CAR T cell research continuum. The fast, simultaneous, multi-parametric single cell analysis of flow cytometry enables the screening of millions of cells in a high-throughput manner for a more comprehensive understanding of the system at play. This allows for flexibility from simple screening of cells transfected with reporter fluorescent proteins all the way to multi-parametric analysis of different cellular traits such as cell health, metabolism, DNA changes, cytokine formation, and other cell phenotypes in a large heterogeneous cell population.

**Attune NxT Flow Cytometer with acoustic focusing technology**

Precision with performance, the Invitrogen™ Attune™ NxT Flow Cytometer is benchtop size and configurable up to 4 lasers, 6–16 parameters, plus superior speed technology up to 10x faster than traditional cytometers, and with clog-resistant engineering. Convert between tubes and plates in seconds, and leverage complete walk-away automation of your 96- or 384-well plates with the robotic automation-capable Invitrogen™ Attune™ NxT Autosampler.

- **Protect precious samples**—clog-resistant design, acoustic focusing technology, high-quality fluids, and a larger flow cell helps prevent the loss of precious samples
- **Go faster**—high-speed flow cytometer with acoustic focusing, top-of-the-line fiber-optic flat-top lasers, and high-quality fluids that allow for true 35,000 events/second acquisition with up to 10x faster (1 mL/min) sample input rates

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**Figure 2. Mouse plasmacytoid dendritic cell (pDC) gating and analysis.** (A) A gate was made on live cells using Invitrogen™ SYTOX™ AADVanced™ Dead Cell Stain (Cat. No. S10274; channel BL3, 640 nm longpass filter), (B) Live cells were then gated on CD19 cells (channel VL1, 450/40 nm bandpass (BP) filter). (C) A 2-parameter plot of CD45R/B220 vs. CD317 was used to detect pDCs (channel BL1, 530/30 nm BP filter; and channel BL2, 574/26 nm BP filter); pDCs were identified as dual B220+/CD317+ (upper right quadrant) and comprise 0.851% of live CD19 cells, which is 0.194% of total splenocytes. A collection rate of 500 μL/min was used to acquire 1.3 million total cells; total acquisition time was 23 minutes—3x faster than the same sample run on a traditional hydrodynamic focusing cytometer.
Flow cytometry antibodies and reagents for studying cancer

Enhance and get more out of your flow cytometry experiments by using the optimal reagents for better results—from sample preparation to reagents for tracking and analyzing cells.

- **Sample prep**—fixation and permeabilization kits, cell lysis and preservation solutions
- **Antibodies**—optimized for flow cytometry
- **Reagents**—cell health, DNA synthesis, cell tracking, cell viability dyes
- **Cell isolation**—with Invitrogen™ Dynabeads™ magnetic beads

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**Cell viability**

Dead cells can give false-positive results as they nonspecifically bind to many reagents. Removing dead cells is a critical step for obtaining accurate flow cytometry results. Choose from a selection of Invitrogen™ viability dyes and assays:

- Fixable viability dyes and nonfixable viability dyes

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**Cell proliferation**

Measuring cell proliferation through the measurement of DNA synthesis is a fundamental method for assessing cell health, determining genotoxicity, and evaluating anticancer drugs. The Invitrogen™ Click-iT™ EdU assays (Figure 3) provide:

- Quantitation of newly synthesized DNA
- Detection without denaturation of DNA
- Compatibility with sensitive R-PE tandems and fluorescent proteins
- Fast detection—in as little as 60 minutes
- An alternative to the cumbersome BrdU assay

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**Detection of cell populations**

Permanently label cells with fluorescent stains to trace generations or divisions without affecting morphology or physiology. Invitrogen™ CellTrace™ cell proliferation kits (Figure 4) offer:

- Labeling *in vitro or in vivo*
- Bright, single-peak staining
- Long-term signal stability
- Options for multiplexing with a variety of lasers

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Cell imaging

Multicolor cellular imaging gives significant information into the cells and the biological systems being studied. In addition to various protein levels, imaging also gives spatial recognition information and other cellular readouts. One such readout is hypoxia. Cellular responses to reduced oxygen (hypoxic conditions) have been linked to a wide range of human pathologies, including tumor development, atherosclerosis, inflammation, and abnormal angiogenesis. Although the importance of hypoxia in inducing these conditions is well known, creating model systems to accurately control the hypoxic conditions is extremely difficult for most researchers. Until recently, to do this effectively, access to elaborate imaging systems that allow maintenance and precise control of temperature, humidity, and gases (CO₂ and O₂) during an experiment was needed. The Invitrogen™ EVOS™ FL Auto Imaging System with Onstage Incubator provides a solution to this situation. This environmental chamber allows for the precise control of oxygen levels, thereby delivering an effective system for researchers to evaluate cellular responses to hypoxia by long-term fluorescence live-cell imaging (Figure 5).

EVOS FL imaging system

The EVOS FL Auto Imaging System with Onstage Incubator is designed to eliminate the complexities of long-term live-cell imaging, allowing researchers to focus on data generation, and not instrument operation and maintenance. It allows for time-lapse cell imaging for long-term monitoring of cell cultures. This flexible, high-performance, and affordable solution for live-cell imaging allows you to:

- **Control**—easily control environmental and image acquisition parameters
- **See more**—create time-lapse images of every well of a 96-well plate
- **Save space**—conserve valuable lab space with a small footprint and sleek design
- **Economical**—helps save money with low-cost ownership and operation

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Figure 5. A549 cells stained with Invitrogen™ Image-iT™ Hypoxia Reagent and exposed to different oxygen levels. (A) 20% O₂. (B) 6% O₂. (C) 2.5% O₂. (D) 1% O₂.
High-content analysis

Like flow cytometry, high-content analysis (HCA) provides single-cell resolution for the quantitative measurements at the single-cell level using multiple fluorescent channels. Unlike flow cytometry, high-content analysis enables the visualization of each cell with spatial resolution (Figure 6). Cell-based screens aid in the identification of the mechanisms of actions in the unexpected disruptions of interconnected pathways and cellular toxicity. As such, cell-based assays are increasingly being used to monitor responses to provide a more accurate reflection of the complexities of the cell compared to the traditional biochemical assays. This enables more rapid evaluation of the cellular efficacy and off-target side effects of different CAR T cell therapies.

**CellInsight CX7 High-Content Screening (HCS) Platform**

The Thermo Scientific™ CellInsight™ CX7 High-Content Analysis Platform is the only HCS platform to incorporate 4-color bright-field, and 7-color fluorescence and confocal imaging. The system provides flexibility and robustness necessary for all HCS workflows and is compatible with existing informatics infrastructure.

- **See more biology**—8-channel LED-excitation for a wider selection of probes
- **Use more techniques**—scan in wide-field, bright-field, and confocal modes in the same assay
- **Read more sample types**—use samples from microscope slides to 1,536-well plates
- **Protect your samples**—high-sensitivity detection and laser-based autofocus help reduce light exposure
- **Save time**—intelligent software and laser-based autofocus enable faster scan times
- **See your data sooner**—analyze data during image acquisition for fast results

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![Figure 6. Human breast cancer tissue stained with the proliferation marker Ki67-DAB and counterstained with H&E.](image)
Cytokine profiling with immunoassays

Cytokine profiling of samples is an important part in immuno-oncology research as it is used to monitor for both cytokine release syndrome (CRS) and the effectiveness of the CAR T cell itself. A great deal is still currently unknown about the cytokines that are released as well as their origins. The monitoring of CRS is the most common off-target severe adverse event of immuno-oncology therapy. Furthermore, the development of CRS is often, but not invariably, associated with clinically beneficial tumor regression. The more that is known about the cytokines and how to control them, the more that might be done with reducing the negative effects of CRS while increasing their anti-tumor benefits.

Detection and quantitation of protein analytes from various biological samples indicate a multitude of biological and pathological events. ELISAs and multiplex immunoassays are routinely used for quantitative assessment of soluble proteins such as cytokines, chemokines, growth factors, and other immunological markers.

**ELISAs and multiplex immunoassays for the Luminex platform**

Invitrogen™ multiplex immunoassays using Luminex™ xMAP™ technology enable the simultaneous quantitation of up to 35 different different analytes using only 25–50 µL of sample in the same amount of time that it takes to perform an ELISA (Figure 7). As immunological and biological systems are comprised of networks of secreted proteins including cytokines, chemokines, growth factors, and other proteins, multiplex immunoassays are an efficient and time-saving method for biomarker profiling of a large set of proteins from a small sample. As such, multiplex immunoassays have proven to be an invaluable tool for the comprehensive study of biological systems.

- **Fast**—quantitate proteins in 3 hours with little hands on time
- **Quantitative**—quantitate up to 35 proteins simultaneously
- **Easy**—as easy to run as an ELISA
- **Expand**—expand or change your biomarker profile as your research advances

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**Figure 7.** Seven individual serum samples were assayed with the Invitrogen™ Human Cytokine Magnetic 25-Plex Panel (Cat. No. LHC0009M) to determine the levels of 25 different cytokines and chemokines simultaneously. Measurements were performed using the Luminex™ 200 system.

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