RaftNote

Applications and protocols for use with CellRaft™ Technology

Functional Genomic Analysis using the CellRaft Technology for Cancer Therapeutics Research

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Abstract

Functional genomic analysis provides a connection between cellular phenotypes, such as drug responsiveness, and their underlying genomic precursors, such as sequence variation or differential gene expression. Here we present data from published work using the CellRaft Technology to couple sophisticated cellular phenotypes with translational molecular data. Welch et al. (2016) reported transcriptomic analysis of pancreatic cancer cells with differential susceptibilities to the small molecule anti-tumor proliferation drug, gemcitabine.[1] Also, Attayek et al., (2017) analyzed T-cell receptor sequences after screening for target cell cytotoxicity.[2] Both studies demonstrate the power of the CellRaft technology for examining complex phenotypes, such as drug sensitivity and cytotoxic activity of T-lymphocytes, and linking these phenotypes with molecular data, such as full transcriptomics or targeted T-cell receptor sequencing. Today, using the CellRaft AIR™ System, investigators continue to pair functional phenotypic data with various molecular read-outs.

The CellRaft™ Technology

The CellRaft Technology allows imaging, sorting, and isolation of living cells in a culture environment which closely replicates standard *in vitro* conditions. Cells are seeded on the CytoSort™ Array where they randomly distribute into microwells. Various experimental interventions are supported in this culture environment including drug treatments, genome edits through CRISPR/Cas9 methods or phenotypic analysis such as proliferation and morphological changes. After imaging and sorting, cells can be isolated using the releasable polystyrene floor of each microwell, a so-called CellRaft. Here we provide data from two laboratories' published studies using the CellRaft Technology to evaluate transcriptomic variations associated with drug sensitivity as well as T-cell receptor sequences from cytotoxic T-lymphocytes demonstrating high degrees of anti-tumor activity. Both studies highlight the power of the CellRaft technology to monitor single cells and pair imaging data with downstream molecular analysis.

Figure 1: CytoSort Arrays come in multiple formats to enable varying numbers of samples and cell titers.

Phenotypic analysis using the CytoSort Array

The CytoSort Array is a microwell array featuring releasable microscale cell culture surfaces, CellRafts, within each well. Multiple formats of the CytoSort Array are available including single array, quad array and 24-array products, each suited to specific laboratory workflows (**Figure 1**). Cells can be cultured, expanded and monitored via the CellRaft AIR™ System's integrated 3-channel fluorescence and brightfield imaging capabilities. The CytoSort

Product	Microwells per Reservoir	Recommended Seeding Titer	Estimated Single Cells
CytoSort 100	40,000	15,000 - 25,000	5,000 - 12,500
CytoSort 200	10,000	4,000 - 6,000	1,500 - 3,000
CytoSort Quad and HexaQuad 100	6,400	2,000 - 3,000	700 - 1,500
CytoSort Quad and HexaQuad 200	1,600	500 - 1,000	150 - 500

Table 1: Specifications on the formats of CytoSort Array with recommended seeding densities and numbers of single cells expected

Array can be coated, incubated and seeded just like a traditional cell culture dish, plate of flask and is compatible with adherent, non-adherent, pluripotent and primary cell types. Because the CytoSort Array replicates standard cell culture consumables, phenotypes observed on the array are comparable to standard *in vitro* conditions. Table 1 describes the number of microwells for each format of the CytoSort Array along with recommended cell seeding densities. Because cells distribute on the array in a Poisson-like distribution, roughly 1/3rd of the cells seeded on the array will reside as single cells within the array's microwells.

Functional Genomics and Drug Screening

In a recent publication, a collaborative team of clinical oncologists, genomics laboratories and bioinformaticians evaluated the CytoSort Array for screening pancreatic cancer cells for drug sensitivity and followed up the screen with transcriptomic analysis to determine differential gene expression between drug-sensitive vs. drug-resistant cells. All of the data and methods are described in detail in the cited report.[1]

CFPAC-1 pancreatic cancer cells were first labeled with a CellTracker Far Red dye. They were then seeded on the CytoSort Array and exposed to 5 nM gemcitabine, a drug known to inhibit cellular proliferation in this pancreatic cancer cell lines. Cells were then monitored for proliferation over time. A representative figure showing the analysis of the proliferation phenotype is shown in **Figure 2**.

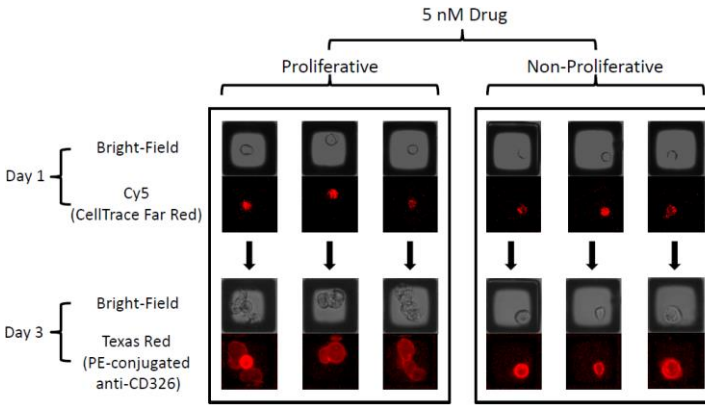

Figure 2: CFPAC-1 cells were monitored for proliferation after exposure to the anti-proliferative compound gemcitabine. After 3 days cells could be characterized as either proliferative (i.e. drug-resistant) or non-proliferative (i.e. drug-sensitive).

After monitoring cells for proliferative phenotypes, they were isolated using the CellRaft Technology. Cells-of-interest were released from the array still attached to the CellRaft microscale cell culture surface and deposited in sample tubes. These were then processed for single cell RNA-Seq using Illumina sequencing instruments. Detailed methods can be found in Welch et al., 2016.

Transcriptomic analysis revealed the up- and down-regulation of several genes already known to be associated with pancreatic cancer cell drug-resistance (heat maps shown in **Figure 3**). These included Immediate Early Response gene X1 (*IEX-1*) which was shown to be downregulated in gemcitabine-resistance cells. CASP4 was also shown to be expressed at a reduced degree compared to drug-sensitive cells. LGALS3 was shown to be upregulated in cells with a drug-resistant phenotype. Previous literature suggests that these differential gene expression patterns are consistent with gemcitabine-resistance and potentially pancreatic cancer drug-resistance more generally.

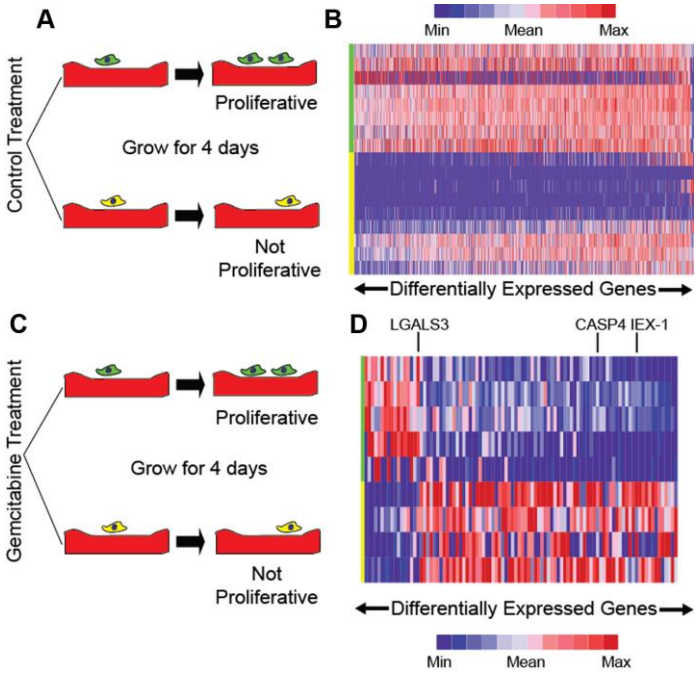

Figure 3: Transcriptomic analysis of proliferative (i.e. drug resistant) and non-prolferative (i.e. drug-sensitive) cells after gemcitabine treatment. Several genes were differentially expressed depending on the proliferation phenotype.

Linking cytotoxic T-cell phenotypes to T-cell receptor sequences

A limited number of endogenous T-cells exhibit cytotoxic effects against cancer cells. New, high-throughput methods for screening large numbers of T-cells for cytotoxic activity against cancer cells, or indeed other pathogens, are essential in identifying T-cell receptor sequences that recognize specific pathogen-related antigens. Characterizing these T-cell receptor sequences which are specific to certain pathogens provides key information in developing autologous and allogeneic T-cell based cell therapies. In a recent paper by Attayek et al., 2017, the CellRaft technology was used to monitor thousands of T-cells for cytotoxicity against dendritic cells expressing the M1p antigen associated with influenza infection.[2] All data and methods are presented in detail in the referenced paper. This study was carried out as a proof-of-principal study for cytotoxic T-cell activity which will lead to experiments revealing anti-cancer T-cell receptor sequences. Dendritic cells were collected from HLA-A*02:01 serotype buffy coats. Purified DCs where then pulsed with the M1p antigen peptide which causes this peptide to be displayed on the HLA-A*02:01 receptor and were stained with Hoechst (UV/Violet spectrum nucleic acid binding dye). CD8+ T-cells were also isolated from HLA-A*02:01 serotype leukapheresis products and stained with CellTracker Far Red. Both cell types were seeded into the CellRaft microwell array. M1p-pulsed DCs were seeded at a density of approximately 30 per microwell and CD8+ T-lymphocytes at approximately 1 per microwell. The microwell seeding strategy is shown in **Figure 4 A-C**.

Microwells containing multiple DCs and only 1 CD8+ T lymphocyte by automated image analysis were tracked over the course of several hours. A field-of-view scan is shown in **Figure 4D** (brightfield channel) and **Figure 4E** (fluorescent merged signal) By including the cell death dye CyTox Green in the culture media, microwells revealing a high level of M1p-pulsed DC cell death (stained blue), and concomitantly including one CD8+ T-cell (stained red) could be easily identified. **Figure 4F** provides an example of a microwell containing multiple CDs, a

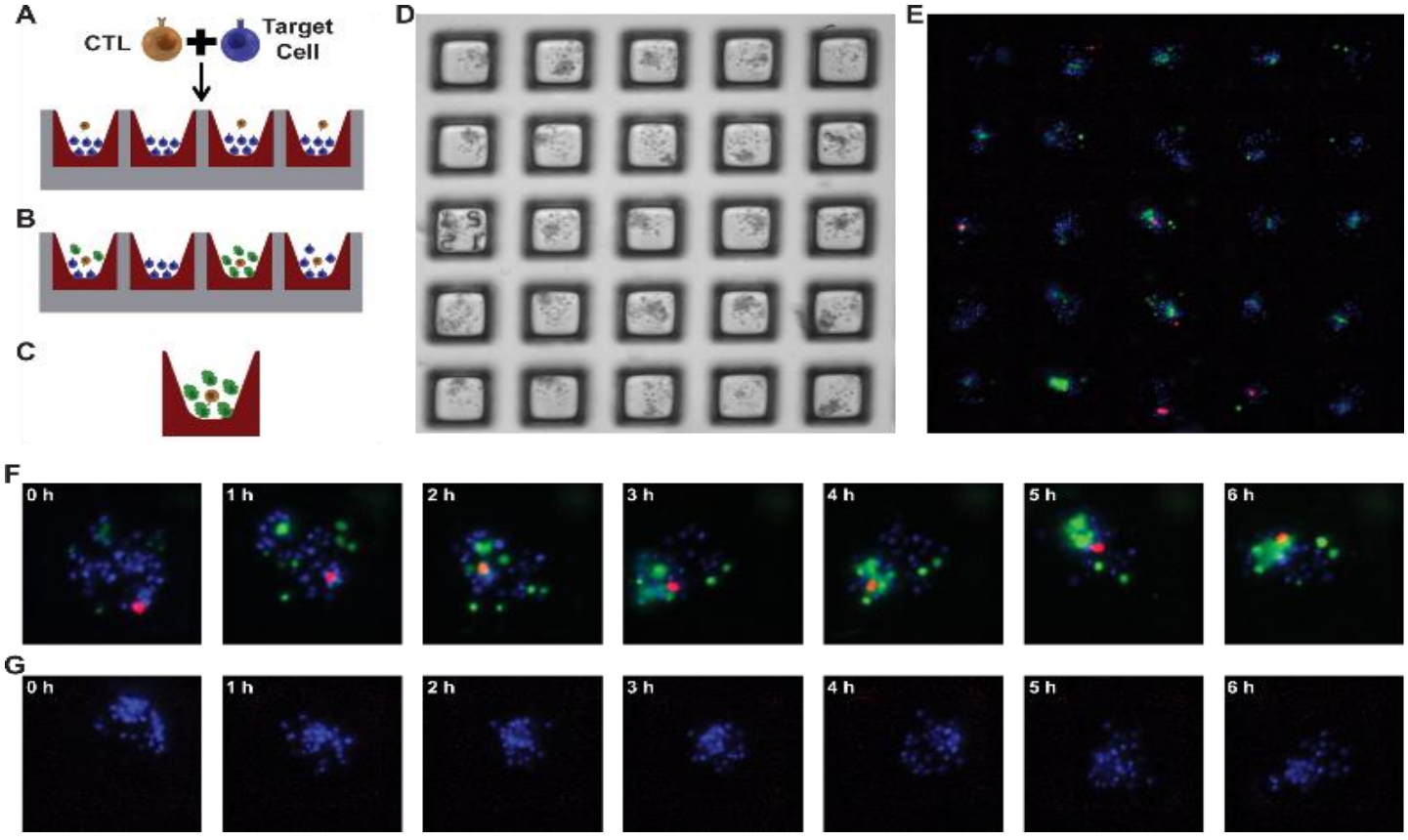

Figure 4: Schematic and imaging data from T-cell screening. A-C: Schematic cell seeding strategy to assure sufficiently microwells contain a single TD8+ T-cell and multiple M1P-positive DC target cells. D, E: Scans of the microwell array during the cell death assay. F: A single microwell showing DC target cells (blue); CD8+ T-cells (red) and the progressive cell death of DCs associated with T-cell cytotoxicity (green). G: The same experiment as F. but without a CD8+ cytotoxic T-lymphocyte.

Clone	TCRα	TCRβ
CTL3-MR-B8	V19-CALSEAGTGGSYIPTF-J6	V19-CASSMFVGQPQHF-J1-5
CTL3-MR-D10	V41-CAVSVEETSGSRLTF-J58	V19-CASSFFHNNEQFF-J2-1
CTL3-MR-F9	ND	V19-CASSIRSSYEQYF-J2-7

Table 2: T-cell receptor sequences identified in CD8+ T-lymphocytes

single CD8+ T-cell and increasing green cell death signal over time. Phenotypically this cell would be described as an efficient killer of Mp1-positive CDs. **Figure 4G** provides a control microwell with no CD8+ T-cell, where very little cell death signal is observed.

Using the releasable microscale culture substrates, CellRafts, found in each microwell, T-cells exhibiting high cytotoxicity toward target cells could be individually isolated. Using relatively standard PCR methods and Sanger sequencing, the T-cell receptor sequences of each phenotypically cytotoxic cell could be determined. This analysis is shown in **Table 2** for several cells that exhibited extremely high degrees of target-cell cytotoxicity.

General Recommendations

The CytoSort array is an ideal tool to capture functional phenotypic data in standard in vitro settings. Imaging cells and collecting them as individuals allows single cell genomic data to be paired with detailed phenotypic data. Automated imaging on the CellRaft AIR System also accelerates functional genomics workflows on the CytoSort Array. Figure 5 shows the AIR System which features an integrated microscope with three fluorescent channels and brightfield capabilities as well as the capability to sort and isolate CellRafts from the CytoSort Array for downstream clonal culture or single cell molecular analysis.

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Literature Cited:

1. Welch, J.D., et al., *Selective single cell isolation for genomics using microraft arrays.* Nucleic Acids Res, 2016.
2. Attayek, P.J., et al., *Identification and isolation of antigen-specific cytotoxic T lymphocytes with an automated microraft sorting system.* Integr Biol (Camb), 2016. **8**(12): p. 1208-1220.

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