RaftNote

Applications and protocols for use with CellRaft Technology

Imaging and Sorting Living Cells Stained with Vital Dyes on Cell Microsystems' CytoSort™ Array and Automated CellRaft AIR™ System

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Abstract

Sorting and isolating single cells is a key sample preparation step in many contemporary workflows including genome editing by CRISPR/Cas9, single cell genomics and differentiation of induced pluripotent stem cells (IPSCs) along with a broad range of other methods. Cell Microsystems has developed the CellRaft AIR™ System to allow imaging-activated cell sorting and isolation of single living cells prior to these downstream analyses. Here, we present optimized methods for staining live cells on the Cell Microsystems CytoSort Array, a microwell array with releasable single cell isolation features called CellRafts. We also describe the use of the automated, bench-top AIR™ System for imaging and isolating vital dye-stained single cells using the CellRaft Technology.

The CellRaft AIR™ System

The AIR™ System (**Figure 1**) is a bench-top instrument comprising an internal microscope with both brightfield and three-channel fluorescent imaging capabilities. The system employs the CytoSort™ Array as a substrate for single cell imaging and culture, as well as the proprietary CellRaft Technology for the isolation and collection of single cells. The AIR™ System software allows users to automatically scan a CytoSort Array for microwells containing individual cells. The system then allows either user-specified selection of cells to be collected as individuals, or cytometric analysis to automatically sort cells based on desirable characteristics according to fluorescent markers. While any type of fluorescent marker can be imaged on the AIR™ System, including transgenic proteins and antibody conjugates, live-cell imaging often requires the use of vital dyes. Here we describe several optimized protocols for vital dye staining of several organellar compartments of cells seeded on CytoSort™ arrays including the nucleus, mitochondria and plasma membrane.

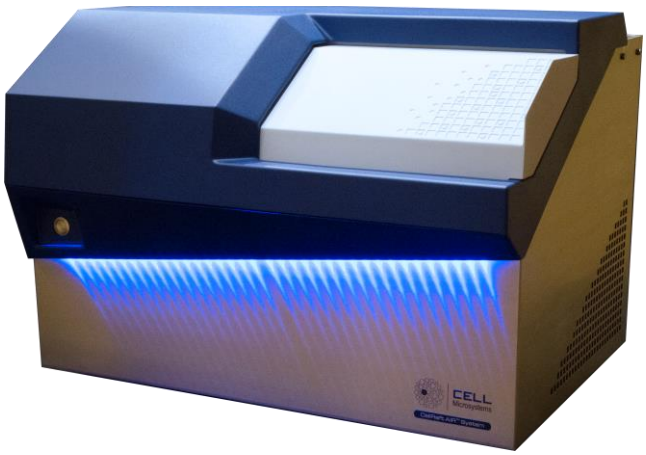

Figure 1: The CellRaft AIR™ System, an automated, bench-top system for single cell imaging, sorting and isolation.

The CellRaft Technology

Cell Microsystems has developed a single cell isolation and recovery platform ideally suited for imaging, sorting and isolation of living single cells. The core technology comprises a disposable microwell array (the CytoSort™ Array; **Figure 2**) on

which cells are seeded and imaged. To isolate single cells, a motorized needle penetrates the resealable elastomeric floor of the array to displace the individual CellRaft from its microwell. The CellRaft material is loaded with magnetic nanoparticles, allowing retrieval of the CellRaft and the attached cell, using a magnetic wand. A schematic of the operation of the CellRaft System is found in **Figure 2**. The AIR™ System relies on the same CellRaft Technology, but automates fluorescent imaging, cytometric sorting and the mechanical release and collection operations to reduce hands-on time and dramatically improve throughput.

The CytoSort™ Array is also amenable to coatings, such as MatriGel, CelTak, and extracellular matrix components such as laminin and fibronectin, allowing the customization of the culture environment for various cells of interest.

The system has been used for a wide range of single cell genomics applications including RNA-Seq [1], whole genome sequencing [2] and single nucleus sequencing [3]. Variations of the technology have also been used for screening T-cells for cancer immunotherapy studies [4] and cloning for genome editing [5].

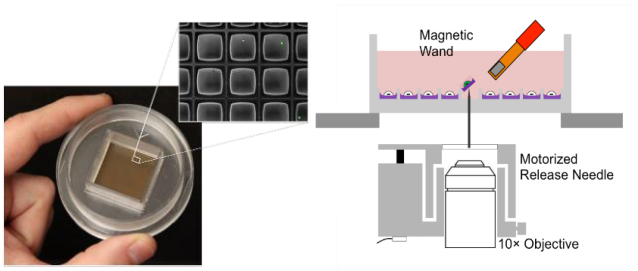

Figure 2*:* The CytoSort™ Array. Raft features are 200X200 microns, with about 10,000 rafts per array. The release needle is positioned on a collar fitted to the imaging objective. The magnetic capture wand is applied by hand to capture and transfer the CellRaft.

Imaging on the AIR™ System

The AIR™ System workflow is schematically depicted in **Figure 3** and relies on imaging of cells seeded on the CytoSort™ Array. The system will count cells using a fluorescent nuclear stain in any of three fluorescent channels. **Table 1** lists the excitation and emission spectra for the AIR™ System optical imaging subsystem. The remaining two fluorescent channels can be used to sort cells using signal intensity histograms and a wide range of gate geometries. Cells can also be isolated in "real-time" with user-specified selection of CellRafts during automated navigation of the CytoSort™ Array. After collection of the selected CellRafts, all fluorescent and brightfield images of collected CellRafts are saved for future analysis and archiving. The system allows the user to set exposure times, gain and other microscope settings using control panels in the AIR™ System software.

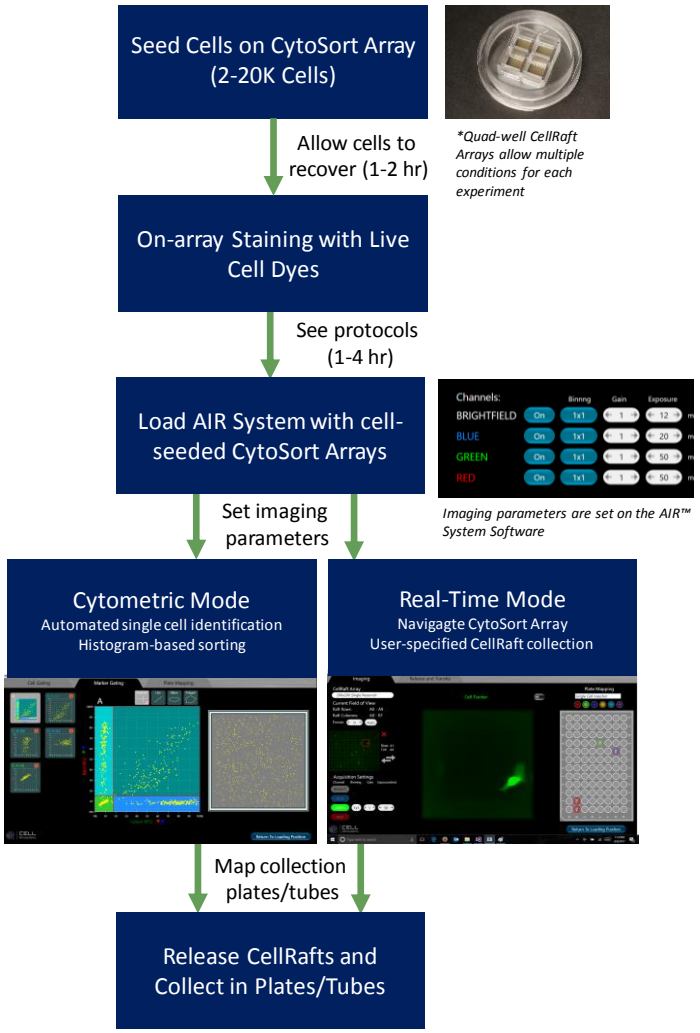

Figure 3: The AIR™ System workflow allows customized imaging settings followed by either "real-time" selection of single cells for isolation, or "cytometric" sorting using histogram-based gating of fluorescent signals prior to isolation of single cells.

Channel	Example Dye	Excitation	Emission
Green	FITC, Alexa488	456-486 nm	497-548 nm
Red	Texas-Red	555-589 nm	602-805 nm
Blue	DAPI, Hoechst	355-371 nm	412-452 nm

Table 1: Excitation/emission spectra for the three fluorescent imaging channels in the AIR™ System.

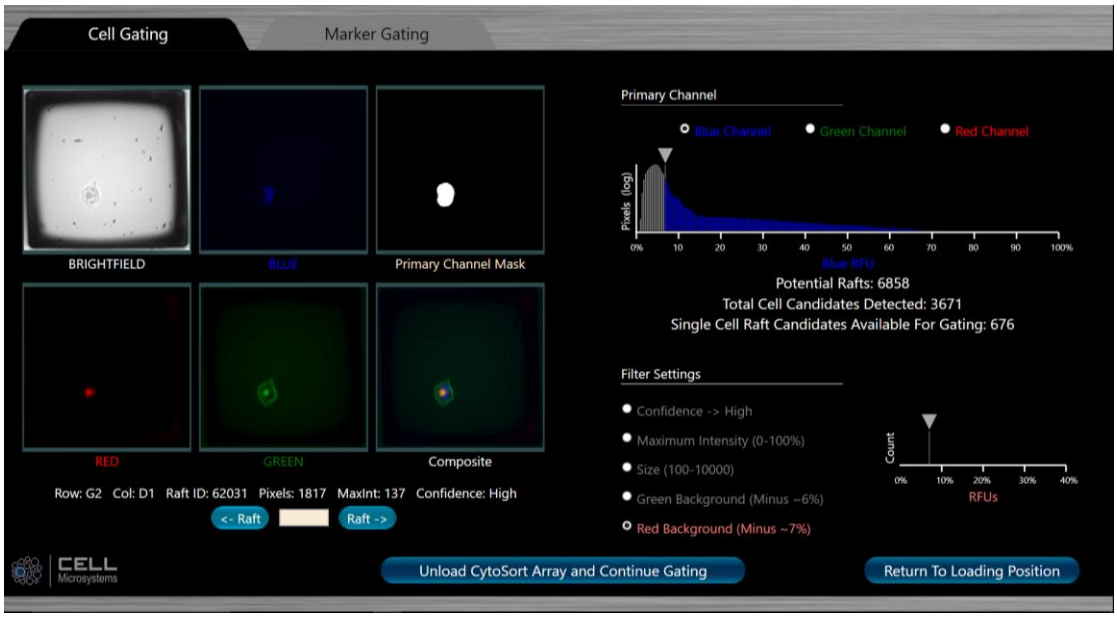

Figure 4: Sample of the AIR™ System software used to set parameters to detect CellRafts containing single cells. After the AIR™ system has imaged the entire CytoSort™ Array, these settings are applied to a fluorescent channel detecting a nuclear dye to identify single cells. Pictured - H1299 cells stained with SYTO 17, Hoechst and Alexa488-Phalloidin.

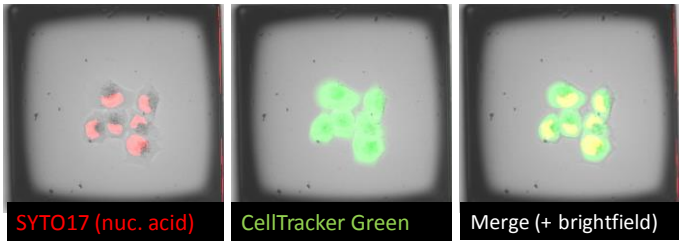

Figure 5: Example of AIR™ System imaging of H1299 cells stained with the nucleic acid stain SYTO 17 which labels nuclei and CellTracker Green which labels the cell broadly.

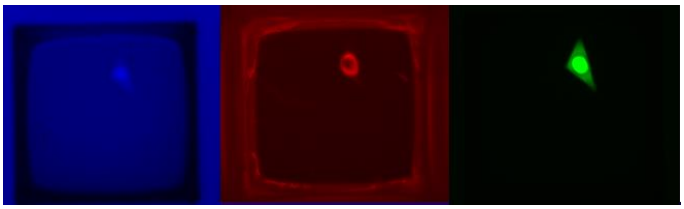

Figure 6: Sample image of a single HeLa cell stained for CellTracker Blue, MitoTracker Red and SYTO 13 (Green). Each dye provided reliable signal and appears to preferentially reveal the appropriate cellular compartment.

Figure 4 provides an example of the "Cell Gating" screen where one channel is used to identify nuclei as a means of automatically identifying those CellRafts on the microwell array which contain only a single cell. By adjusting the nucleus-detecting "Primary Channel" threshold as well as the filter settings (confidence, size, etc.), the detection of nuclei can be optimized for subsequent single cell sorting and collection via automated algorithms.

Organellar localization of live cell dyes

Many live cell dyes label a specific organellar compartment, such as the nucleus (SYTO™ Dyes) or mitochondria (MitoTracker™ dyes) and others are used to track cells broadly (CellTracker™ dyes). Here, we examined two cell types, H1299 and HeLa, for subcellular localization of various live cell dyes. It should be noted that the AIR™ System automatically identifies single cells on the CytoSort™ Array by detecting nuclei. A nuclear stain is therefore highly recommended for automated single cell detection using the AIR™ System. Based on the data shown here, we recommend several dyes for this purpose including Hoechst, SYTO 17 (**Figure 5**; red-emitting nucleic acid dye) and SYTO 13 (**Figure 6**; green-emitting nucleic acid dye). The choice of nuclear dye should be based on: its utility in live vs. fixed cells and the downstream application. Hoechst and DAPI are known to interfere with assays such as PCR, and so a SYTO dye may be preferred since they do not interfere with downstream molecular assays [3]. Other live cell dyes have also been tested and are compatible with both the CytoSort™ Array and the AIR™ System. These include CellTracker and MitoTracker dyes (**Figure 5 and 6**).

Protocols for on-array vital dye staining

Both H1299 and HeLa cells were used to optimize on-array staining protocols using vital dyes on the CytoSort™ Array. **Table 2** provides an overview of the dye-specific parameters. These include the concentration of the dye used and exposure times for imaging CytoSort™ Arrays on the AIR™ System. All cells were seeded on the CytoSort™ Array at 4-6 thousand cells per array (which comprises approximately 10,000 microwells with a releasable CellRaft at the bottom of each microwell). Cells were stained at the dye concentrations listed in **Table 2** for 30 min and were then washed once with 1X PBS prior to imaging. We have also found that coating the CytoSort™ Array surface with collagen IV promotes cell spreading for more detailed imaging.

Dye	Concentration	Exposure Time
CellTracker™ Green CMFDA	5 µM	20 ms
CellTracker™ Blue	5 µM	100 ms
SYTO® 13 Green	5 µM	2 ms
SYTO® 17 Red	5 µM	15 ms
MitoTracker® Red FM	500 nM	50 ms
Hoechst 33342	1.6 µM (1 µg/mL)	20 ms

Table 2: Live cell staining protocol parameters for on-array staining and imaging using the AIR™ System.

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