RaftNote

Applications and Protocols for Use with CellRaft Technology

CELL Microsystems

Methods for Preparing Cryopreserved Neural Tissue Samples for Single Nucleus Sequencing (SNS) Using the CellRaft™ Technology

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Abstract

Single cell genomic analysis has already impacted a range of life science fields, including tumor clonality, neuronal mosaicism, and drug resistance mechanisms. These methods rely on access to relatively fresh samples, ensuring that a maximum amount of high quality nucleic acid can be extracted from a given cell. Conversely, translational research depends on the availability of human samples which are difficult to acquire in large numbers as fresh samples. As an alternative to single cell sequencing, single nucleus sequencing (SNS) has enabled genomic analysis using the plentiful cryopreserved samples found in various biobanks and other facilities. The CellRaft Technology uniquely enables the sorting and isolation of single nuclei for downstream analysis. Here we provide optimal methods for imaging nuclei on CytoSort Arrays, and downstream molecular biology protocols for the amplification and preparation of nucleic acid prior to sequencing.

Translational Single Cell Analysis using SNS

Translational research relies on patient samples which are often stored in biobank facilities. Nucleic acid extraction from preserved samples often results in compromised integrity rendering single cell analysis challenging. To address this shortcoming, many investigators are turning to single nucleus isolation prior to molecular analysis. Here, we provide details on methods for isolating nuclei from cryopreserved neural tissue samples for subsequent genomic analysis. The methods for SNS described here using the CellRaft Technology were developed by the laboratory of Mike McConnell, PhD of the University of Virginia. [6] The workflow steps are shown in **Figure 1**. Once nuclei from specific cell types have been isolated (in this case neurons), the nuclei are lysed and the DNA is amplified and prepared for sequencing. The CellRaft Technology allows nuclei to be isolated and sorted for cell type-specific markers prior to downstream molecular analysis.

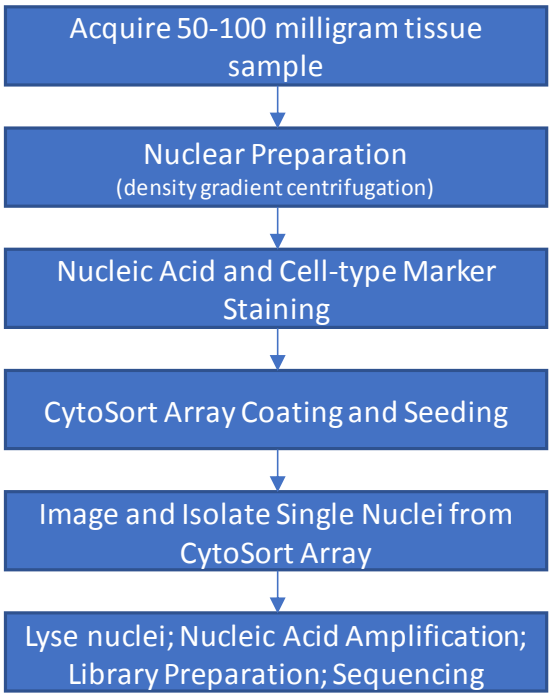

Figure 1: Schematic of the SNS whole genome sequencing workflow using CellRaft Technology.

The CellRaft Technology

Cell Microsystems has developed a single cell isolation and recovery platform ideally suited for imaging, sorting, and the isolation of single nuclei for SNS. The core technology comprises a disposable microwell array (the CytoSort™ Array; **Figure 2**) on which nuclei can be seeded and imaged. To isolate single nuclei, 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 nucleus, 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, Cell-Tak, 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].

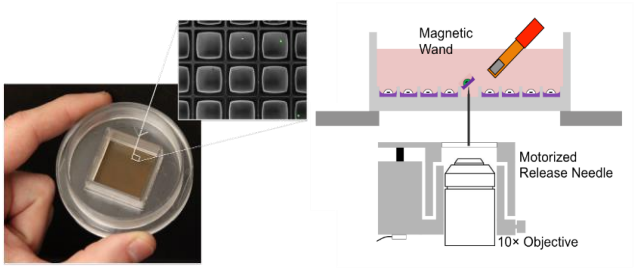

Figure 2*:* The CytoSort™ Array. Raft features are 200X200 microns, with about 10,000 rafts per array. The release needle is positioned on a collar fitted to the imaging objective. The magnetic capture wand is applied by hand to capture and transfer the CellRaft.

Preparing, Staining, and Isolating Neuronal Nuclei

Tissue was acquired from the NIH NeuroBioBank for these studies. From 50-100 mg of tissue sample, nuclei were isolated using density gradient centrifugation (OptiPrep™). To ensure the isolated nuclei contain genomic material and distinguish neuronal from non-neuronal nuclei, the preparation was stained for both DNA content and a neuron-specific nuclear-splicing factor, NeuN[6-8]. Specifically, bulk nuclei were suspended in PBS containing 0.1% Tween and 1.0% BSA and incubated for 1 hour at 4°C with gentle agitation. SYTO13 nucleic acid stain (50 nM) and AF555-conjugated anti-NeuN antibody (1:500, 20 µg/mL) were then added and incubated with gentle agitation overnight at 4°C. To create a 'sticky' surface on the rafts, Cell-Tak (Corning) was applied to the CytoSort Array using 1 mL alkalized PBS (PBS, 2.5 mM NaOH, 15 µg/mL) according to the basic absorption coating protocol detailed by Corning [9]. The Cell-Tak solution was then removed and the CytoSort Array was rinsed three times with ddH_2O. Between 2,000 and 40,000 nuclei in 2 mL of PBS were then added to the CytoSort Array to achieve a random distribution of nuclei across the array. Spreading less than 2,000 nuclei results in too sparse a distribution to efficiently locate and retrieve microwells with single nuclei, and spreading over 40,000 results in multiple nuclei per well. At this stage, nuclei can be imaged using both brightfield (**Figure 3A**) and standard fluorescent microscopy to identify SYTO13+/NeuN+ nuclei (**Figure 3B**).

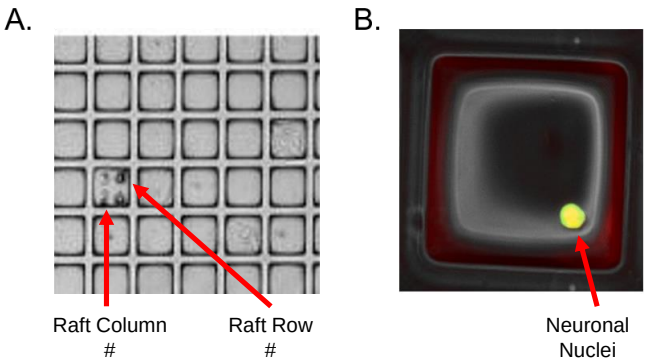

Figure 3: A: Brightfield image of the CytoSort Array showing the Row/Column addressing system; B: A NeuN+ nucleus on a CellRaft.

Isolation of Nuclei for Downstream Analysis

To transfer nuclei from the raft surface to a PCR tube, a well containing a neuronal nucleus (NeuN positive) was identified manually using the appropriate fluorescent filters. The CellRaft of interest was then positioned above the release needle (empirically determined on the microscope after CellRaft System installation. For compatibility

of microscopes, contact Cell Microsystems, Inc. at info@cellmicrosystems.com).

Under brightfield, the plastic retrieval wand containing a magnet to capture the paramagnetic nanoparticle-containing CellRaft was placed over the microwell. The needle on the release device was then activated, actuating the needle through the underside of the CytoSort Array to free the CellRaft from its microwell. The CellRaft then attached to the tip of the retrieval wand. To release the well from the wand, the tip of the wand holding the CellRaft was placed into a PCR tube containing appropriate buffer. The presence of the CellRaft can be confirmed visually by microscope. Rinsing the retrieval wand in sterile PBS or ethanol can also be performed to maintain desired levels of sterility between collection operations.

Alternative Nucleic Acid Dyes for Nuclear Sorting

In the workflow described here, nuclei are sorted based on two criteria: 1) positive for nucleic acid signal based on SYTO 13 staining, and 2) NeuN positive to indicate a neuronal cell type. Given the breadth of single cell analyses undertaken by translational researchers, multiple stains for cell phenotypes may be required for an experiment. To accommodate as much flexibility as possible, Cell Microsystems has evaluated nucleic acid dyes in all channels supported by the new CellRaft AIR System. Ensuring a nuclear stain is compatible with all three fluorescent channels of the AIR System provides sufficient flexibility to allow cell type-specific markers or other phenotypic stains to be used in the remaining channels. **Figure 4** shows sample images of HeLa cells stained with Hoechst (UV excitation channel), SYTO 13 (yellow-orange excitation channel) and SYTO 17 (red excitation channel). Preliminary data using a violet excitation LED on the AIR System also indicates that SYTO 41 is an effective nuclear stain on CytoSort Arrays. Protocols for staining cells with these dyes, as well as other vital dyes such as CellTracker and MitoTracker, can be found at the Cell Microsystems website. A RaftNote describing live-cell staining and imaging is available here.

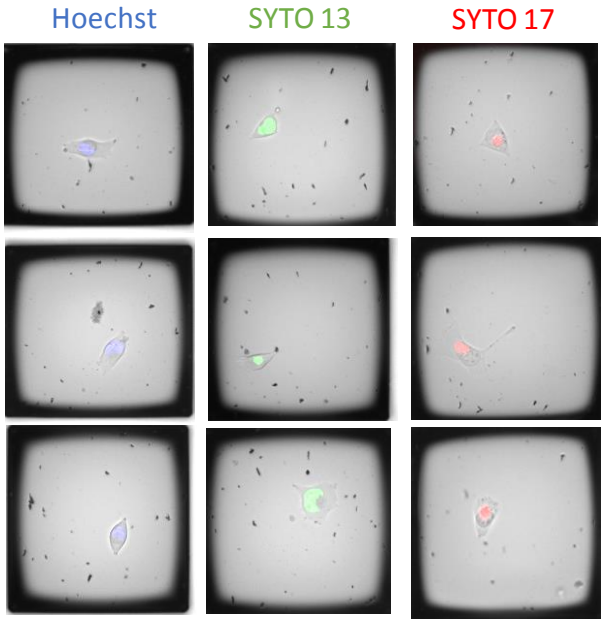

Figure 4: Staining nuclei with various nucleic acid dyes on the CytoSort Array. Images were taken using the automated AIR™ System from Cell Microsystems

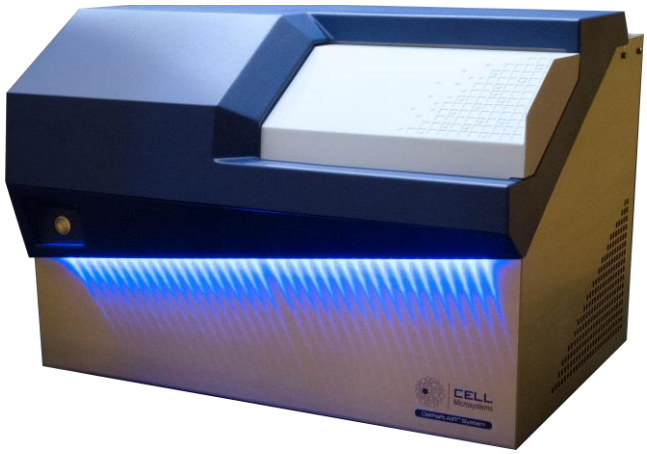

Figure 5: The CellRaft AIR™ System, an automated, bench-top system for single cell imaging, sorting, and isolation.

While the data and methods describe previously rely largely on the manual CellRaft System, the AIR System also facilitates the same workflow using an automated, bench-top instrument with an integrated microscope, fluorescent excitation/emission, and software-based user interface. The AIR System is shown in **Figure 5** and more details about its capabilities can be found here.

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