Automated in situ purification of primary rat brain microvascular endothelial cells

Introduction

Primary cells can potentially provide more physiologically relevant systems for study in discovery biology and drug discovery applications, as compared to immortalized cell lines. However, it is very difficult to obtain primary cell preparations with sufficient purity due to limited cell numbers, and particularly with adherent cell populations. Most current approaches to purifying cells, such as flow cytometry, require enzyme treatment to suspend cells for processing, generally leading to reducing or removing important cell surface markers. Substrate-attached differentiated cell types are particularly challenging as manipulations can directly alter their biology, and forcing them into suspension leads to altered gene expression and decreased cell survival.

The LEAP™ (Laser-Enabled Analysis and Processing) system has been developed to address current limitations in cell purification. The LEAP Cell Purification Application operates through the laser-mediated in situ elimination of undesired cells without physically manipulating the cells that are preserved. LEAP has been used for high-throughput laser-mediated cell elimination for general cell purification (Koller et al, 2004), as well as purification of cells based on direct measurement of antibody secretion by individual cells (Hanania et al, 2005).

The results shown here establish that the LEAP Cell Purification Application results in significantly improved purification of adherent primary brain microvascular endothelial cells with high yield and viability, demonstrating the capability of LEAP to address key needs in primary cell purification applications.

**LEAP System Features & Benefits**

<table>
<thead>
<tr>
<th>Feature</th>
<th>Description</th>
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<tr>
<td>Whole well imaging</td>
<td>All cells in the well can be analyzed and processed</td>
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<td>Purify specific subpopulations of cells</td>
<td>in situ</td>
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<td>Limited plate movements</td>
<td>Work effectively with attached and non substrate-attached cells</td>
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<tr>
<td>Image magnifications</td>
<td>3X, 5X, 10X, or 20X</td>
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<td>Combinations of excitation and emission wavelengths</td>
<td>8 excitation and 8 emission wavelengths</td>
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Figure 1. Impure preparation of primary rat brain microvascular endothelial cells stained with CellTracker Green and Dil-Ac-LDL (left). Contaminating fibroblasts are identified by arrows (upper inserts). Contaminating cells were destroyed using the 532 nM laser of LEAP, leaving behind a pure population of Dil-Ac-LDL stained endothelial cells (center). After 72 hrs, extensive outgrowth of a pure endothelial cell population was observed (right).
Validation Approach

Microvascular endothelial cells represent an example of cells that are difficult and time-consuming to purify (Scott et al., 1993). Brain microvascular endothelial cells, in particular, are of great scientific interest due to their role in immune cell trafficking, blood-brain barrier regulation, and immune response regulation in the CNS. Microvascular cells are usually prepared by an enzymatic digestion of tissue, followed by density centrifugation, flow cytometry or bead capture, and then are propagated in specific culture media formulations. The utility of flow cytometry or bead capture is limited for these cells due to: (1) the very low numbers of cells available from primary rat brains; and (2) the significant sensitivity of these cells to trypsinization and being in suspension. At best, current procedures generate cultures of ~80% purity.

Results

The LEAP Cell Purification Application was used to automatically purify primary rat brain microvascular endothelial cells in the attached state. Cells were seeded at 400 cells per well in 384-well C-lect™ culture plates that had been pre-coated with 10 μg/ml fibronectin. Cells were allowed to attach and grow overnight, loaded with 10 μM Dil-Ac-LDL for 4 hours, and stained with 2.5 μM CellTracker™ Green for 1 hour (Fig. 1). Just before processing, Cyntellect’s Phototherm™ sensitizer was added to each well to increase laser energy absorption. Only endothelial cells became stained with Dil-Ac-LDL, whereas all cells were stained by CellTracker Green. Contaminating fibroblasts could be identified as large flat cells that did not label with Dil-Ac-LDL.

The LEAP Cell Purification Application was used to gate on all CellTracker Green positive cells that were also positive for Dil-Ac-LDL (red), causing these cells to be spared from laser irradiation. Optimum processing conditions were identified which led from a starting purity of 60.1% to a final 99.9% purity of Dil-Ac-LDL positive endothelial cells (Fig. 1). To ensure destruction of all contaminating cells, gates were set to target some cells that contained the lowest levels of Dil-Ac-LDL staining, resulting in the loss of a few endothelial cells as well. A small trade off of slightly reduced yield (by ~15%) was made to achieve 99.9% purity in this example.

The fully automated processing capability of LEAP was used to process 90 wells of a 384-well plate, requiring an average 6 seconds per well. Endothelial cell purity of 99.9% with an average yield of 84.6% was obtained. Notably, cell growth rates in processed wells were not affected by laser processing, and were identical to unprocessed control cells (data not shown).

To verify that laser purification had no effect on endothelial cell function cells we tested for differentiation in an angiogenesis assay. Purified cells showed an increased incidence of differentiation to higher order structures (Fig. 2). The standard 96-well angiogenesis assay format was also successfully translated to a 384-well format for greater throughput.

Conclusion

Automated, high-efficiency, high-yield cell purification of primary rat brain microvascular endothelial cells was achieved with extremely low initial cell numbers, and was done in an in situ manner avoiding trypsinization and the physical manipulation of desired cells. Purity was maintained after outgrowth confirming removal of contaminating cells. Growth rates of laser-processed cells were equivalent to untreated cells and showed improved characteristics in a standard angiogenesis differentiation assay. The LEAP Cell Purification Application enables efficient purification of cells from small tissue samples, even in the attached state, enabling potentially novel biological approaches which rely on use more relevant primary cell samples.

References

Hanania et al, Biotechnology and Bioengineering, 91(7), 2005
Scott and Bicknell, J Cell Sci., 105 (Pt 2):269-73, 199