Laser Capture Microdissection of Cells Labeled with Enhanced Green Fluorescent Protein

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Abstract

This application note describes a Laser Capture Microdissection (LCM) method for the isolation of cells expressing enhanced green fluorescent protein (EGFP) from frozen sections of embryonic tissue. The application of this methodology was used to facilitate retrieval of RNA from mouse embryonic neural crest cells that could not be distinguished by morphology, histological stains or labeling by fluorescent antibodies in a heterogeneous tissue. Specificity of the LCM of the neural crest cells was confirmed by quantitative real-time PCR of neural crest gene markers.

Introduction

The emergent technology of Laser Capture Microdissection (LCM; Arcturus; Emmert-Buck et al., 1996; Simone et al., 1998) is used to isolate specific cell types from tissue sections for downstream molecular analyses. In traditional applications, LCM is used to isolate cells that are distinguished by morphology, histological stains, or labeling by fluorescent antibodies from a heterogeneous population of cells in a tissue section. In this Application Note, an adaptation of this technique for the microdissection of cells genetically labeled with enhanced green fluorescent protein is described (EGFP; Bhattacherjee et al., 2004). EGFP is a recombinant protein originally derived from the jellyfish, *Aequoria victoria*, which has the advantage over other reporters of not requiring cell/tissue fixation for visualization. EGFP fluoresces green when excited by light of 488 nm and may be visualized by epifluorescence microscopy using standard fluorescein isothiocyanate filters (Chalfie et al., 1994) under wavelengths that are not deleterious to living tissues. Since EGFP consists of ~200 amino acid residues, it may be expressed either as a tag at the c-terminus of an endogenous protein (a fusion protein) or as a free peptide in the cytoplasm. Owing to the high solubility of EGFP when expressed as a free protein, fixation is required to prevent leaching of the protein and retain fluorescence after cryosectioning. Recently, chromosomal expression of EGFP from transgenic reporter constructs has been adopted for use as a cell type-specific label in transgenic mice. The combination of cell type-specific EGFP expression with the powerful technique of LCM allows the isolation of RNA from cell types, which are otherwise morphologically similar to surrounding cells or which cannot be detected by immuno-labeling. This method describes the isolation of an embryonic stem cell population, the neural crest, that has been genetically labeled with EGFP and the subsequent extraction of mRNA and amplification of neural crest cell gene markers (Bhattacherjee et al., 2004).

Equipment and Reagents

This protocol requires the following reagents:

- ❑ Phosphate buffered saline (PBS)
- ❑ 4% Paraformaldehyde (PFA) dissolved in PBS
- ❑ Silane Prep Slides or equivalent (Sigma Aldrich, St. Louis, MO, Cat. # S-4651)
- ❑ CapSure HS LCM Caps (Arcturus, Cat. # LCM 0204)
- ❑ Optimal cutting temperature compound (OCT - Tissue-Tek, Cat. # 4583)
- ❑ PicoPure™ RNA isolation kit (Arcturus, Cat. # KIT0204)
- ❑ RiboAmp™ RNA amplification kit (Arcturus, Cat. # KIT0203)
- ❑ Superscript II™ reverse transcriptase (Invitrogen Life Technologies, Inc., Carlsbad, CA, Cat. # 18064-022)

Equipment and Labware

The following laboratory equipment is required:

- ❑ PixCell IIe® LCM Instrument equipped with Fluor 300 epifluorescence optics optimized for EGFP
- ❑ TaqMan™ ABI Prism 7000 Sequence Detection System (Applied Biosystems, Foster City, CA)

Special Considerations

Excessive fixation of the tissue prior to embedding prevents efficient extraction of RNA from captured cells. Illumination of tissue sections under epifluorescence optics should be minimized in order to reduce photo-bleaching and prolong EGFP fluorescence.

Method

CRYOSECTIONING AND LCM

1. Fix freshly dissected tissues in 4% paraformaldehyde in phosphate buffered saline (PBS) for 5 min. Mild fixation by 4% PFA retains EGFP within the cells during embedding and sectioning without affecting subsequent RNA extraction. Wash the tissue twice in PBS for 5 min, then freeze in OCT compound on dry ice.

2. Pre-clean the internal surfaces of the cryostat with 100% ethanol. Perform cryosectioning onto silanized glass slides in a cryostat at –19 °C using a fresh disposable low-profile blade under RNase-free conditions. Mount sections onto slides at room temperature but subsequent storage should be inside the cryostat (short term) or at –70 °C (long term).

3. Process tissue sections for LCM within 2 days of cryosectioning. Allow mounted tissue sections to thaw for no longer than 20 seconds before beginning dehydration. Dehydrate sections in 70%, 95% and 100% ethanol for 30 seconds each, followed by 5 min in xylene. Air-dry sections for 2 minutes before commencing LCM.

4. Visualize EGFP-expressing cells in the tissue sections using the EGFP-optimized emission filters with an integration setting of 0.12-0.2 using an Arcturus PixCell IIe® system equipped with epifluorescence optics.

5. A spot size of 7.5 µm with power settings of 60-70 mA and a pulse duration of 1.3 msec ensured efficient capture of cells.

6. The laser spot overwhelms green fluorescence, so comparison of an image map acquired under fluorescence with the live camera image (under brightfield or fluorescence) during LCM allows accurate capture of EGFP-expressing cells

7. After completing LCM, perform extraction, purification and amplification of RNA using the Arcturus PicoPure™ and RiboAmp™ kits according to the manufacturer's protocols.

8. Synthesize cDNA from amplified RNA with random hexamer primers and Superscript II™ reverse transcriptase to confirm expression of cell type-specific gene targets by quantitative real-time PCR (Bhattacherjee et al., 2004).

Results

During dissection, embryos in a litter that express EGFP in a pattern specific to neural crest cells can be identified by fluorescent stereo microscopy prior to fixation and embedding in OCT (Figure 1). EGFP-labeled neural crest cells can be identified and captured by LCM from partially fixed cryosections of embryonic tissue (Figure 2). Quantitative Real-Time PCR of the RNA extracted from these captured cells resulted in amplification of neural crest cell gene markers (Crabp1, Dlx5, Msx1, Slug), while expression of Engrailed-1 (En1), a gene that is not expressed in the neural crest cells was not detected (Table 1). These results demonstrate the utility of LCM to isolate specific EGFP-expressing cell populations

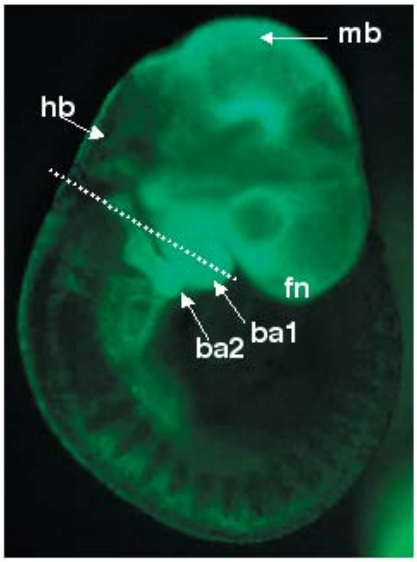

Figure 1. EGFP-labeled neural crest cells in transgenic mouse embryos.
A 9.5-dpc (days postcoitum), transgenic embryo photographed under epifluorescence optics showing EGFP expression in the neural crest cells of the first and second branchial arches (ba1 and ba2), and in the neural crest cells of the frontonasal region (fn), as well as in Wnt1 expressing cells of the midbrain/hindbrain (mb and hb). The dashed line indicates the plane of the section shown in Figure 2.

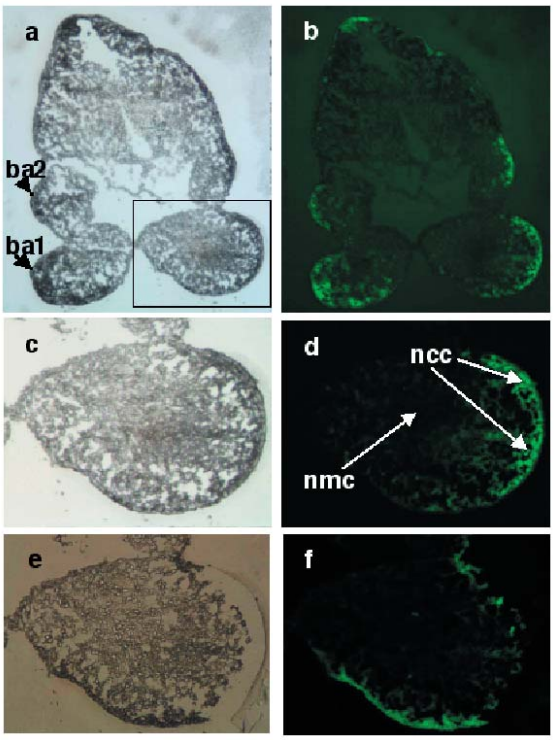

Figure 2. LCM of EGFP-labeled cranial neural crest cells from the first branchial arch of a 9.5-dpc transgenic mouse embryo.
Tissue sections were visualized under brightfield (a, c and e) and epifluorescence (b, d and f) optics. (a-b) Transverse section of a 9.5-dpc embryo through the region of the first and second branchial arches (ba1 and ba2) (40X) (c-f) Higher magnification (200X) of the first branchial arch region outlined in (a). (c-d) First branchial arch prior to LCM showing peripheral, fluorescent neural crest cells (ncc) and the non-neural crest mesenchymal core (nmc). (e-f) The identical branchial arch, photographed after LCM of the peripheral EGFP-labeled neural crest cells.

Table 1. Expression of neural crest marker genes by LCM-isolated, EGFP-labeled neural crest cells.[1]

MARKER GENE	GENE AMPLIFICATION[2]	C_t VALUE[3, 4]
Msx1	+	32.1
Slug	+	30.6
Dlx5	+	30.2
Crabp1	+	22.1
Gapdh	+	21.2
En1[5]	-	No amplification

[1]Expression of neural crest gene markers was determined using TaqMan™ quantitative real-time PCR.
[2]"+" indicates detectable levels of gene expression; "-" indicates undetectable levels.
[3]Neural crest cDNA samples were prepared and subjected to QRT-PCR for each target gene in triplicate; mean Ct values are reported.
[4]Negative methodological control reactions, which lacked reverse transcriptase, did not amplify any detectable product.
[5]En1 - a negative control gene that is known not to be expressed by neural crest cells.

from tissue sections of transgenic mouse embryos that can be used for subsequent molecular analyses.

References

1. Emmert-Buck MR et al., Laser Capture Microdissection. Science 274:998-1001, 1996.
2. Simone, NL et al., Laser-capture microdissection: opening the frontier to molecular analysis. Trends Genet 14:272-6, 1998.
3. Bhattacherjee et al., Laser capture microdissection of fluorescently labeled embryonic cranial crest cells. Genesis 39:58-64, 2004.
4. Chalfie M et al., Green fluorescent protein as a marker for gene expression. Science 263:802-5, 1994.

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