

Video Article

# Heterokaryon Technique for Analysis of Cell Type-specific Localization

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## Abstract

A significant number of proteins are regulated by subcellular trafficking or nucleocytoplasmic shuttling. These proteins display a diverse array of cellular functions including nuclear import/export of RNA and protein, transcriptional regulation, and apoptosis. Interestingly, major cellular reorganizations including cell division, differentiation and transformation, often involve such activities<sup>3,4,8,10</sup>. The detailed study of these proteins and their respective regulatory mechanisms can be challenging as the stimulation for these localization changes can be elusive, and the movements themselves can be quite dynamic and difficult to track. Studies involving cellular oncogenesis, for example, continue to benefit from understanding pathways and protein activities that differ between normal primary cells and transformed cells<sup>6,7,11,12</sup>. As many proteins show altered localization during transformation or as a result of transformation, methods to efficiently characterize these proteins and the pathways in which they participate stand to improve the understanding of oncogenesis and open new areas for drug targeting.

Here we present a method for the analysis of protein trafficking and shuttling activity between primary and transformed mammalian cells. This method combines the generation of heterokaryon fusions with fluorescence microscopy to provide a flexible protocol that can be used to detect steady-state or dynamic protein localizations. As shown in Figure 1, two separate cell types are transiently transfected with plasmid constructs bearing a fluoroprotein gene attached to the gene of interest. After expression, the cells are fused using polyethylene glycol, and protein localizations may then be imaged using a variety of methods. The protocol presented here is a fundamental approach to which specialized techniques may be added.

## Video Link

The video component of this article can be found at <https://www.jove.com/video/2488/>

## Protocol

### 1. Transfection of Cell Lines

#### Transfection Method 1

(Lonza Nucleofection for increased primary cell transfection efficiency)

1. Cells should be of recent passage and in log phase growth prior to transfection. Wash cells in phosphate buffered saline (PBS) and harvest cells by trypsinization.
2. Collect cells by centrifugation at 1500 x g for 5 minutes.
3. Add sterilized cover slips to a 6-well plate. Cover slips may be coated for enhanced cell adhesion. Place 1.5 mL of culture media (in this case Dulbecco's modified Eagle's medium (DMEM), 10% fetal bovine serum (FBS)) into each well and equilibrate the media in the incubator.
4. Harvest the cells by trypsinization and resuspend in a minimal volume of phosphate buffered saline (PBS).
5. Count the cells and centrifuge the correct amount of resuspension to provide  $\sim 5 \times 10^5$  cells per sample.
6. Resuspend the cells carefully in 100  $\mu$ L room temperature Nucleofector solution per sample.
7. Combine 100  $\mu$ L of cell suspension with 5  $\mu$ g plasmid DNA and transfer the sample to a Nucleofector Cuvette being careful not to include air bubbles.
8. Select the appropriate Nucleofector Program (this may require previous testing to establish optimal transfection efficiency with minimal mortality).
9. Insert the cuvette containing the cell/DNA suspension into the Nucleofector Cuvette Holder and begin the selected program.
10. Upon completion, remove the cuvette and add  $\sim 500$   $\mu$ L of pre-equilibrated culture media to it (taken from the 6-well plate).
11. Immediately transfer the sample into the 6-well plate with final volume of 1.5 mL per well. Cells should be plated in either mixed populations or separately, for controls.

## Transfection Method 2

(Qiagen Effectene transfection for cell lines)

1. Prepare transfection complexes using the Qiagen Effectene Reagent by first adding 0.8  $\mu\text{g}$  of each plasmid DNA sample to 200  $\mu\text{L}$  EC buffer.
2. Add 6.4  $\mu\text{L}$  enhancer reagent to each sample, mix briefly by flicking the tube, and incubate for 2 minutes at room temperature.
3. Add 20  $\mu\text{L}$  Effectene reagent to each sample, mix by flicking the tube, and incubate for 15 minutes at room temperature.
4. During this incubation, prepare the two cell lines of interest for transfection. Wash recently passaged cells (<80% confluence) once in phosphate buffered saline (PBS). Add back 1.5 mL fresh warmed and equilibrated growth medium (in this case Dulbecco's modified Eagle's medium (DMEM), 10% fetal bovine serum (FBS)).
5. Once the transfection complexes have incubated for 15 minutes, transfer 1.2 mL of media to each sample. Mix by pipetting, and immediately transfer ~700  $\mu\text{L}$  of the complexes to each of two wells in the 6-well plate. Add dropwise, and mix by gently by swirling the plate. Allow cells to transfect for at least 3 hours (may vary with cell type).
6. Add sterilized cover slips to a new 6-well plate. Cover slips may be coated for enhanced cell adhesion.
7. After the cells have transfected, wash cells twice with PBS and harvest the cells by minimal trypsinization by adding approximately 100  $\mu\text{L}$  trypsin solution to each well, briefly agitating the plate to fully coat each well, and immediately aspirating off the trypsin. Once the cells have lifted, add 1.5 mL fresh media.
8. At this point, cells should be plated in either mixed or separate populations (for controls) onto the sterile cover slips.
9. Allow cells to recover for 12 hours.

## 2. Cell Fusion

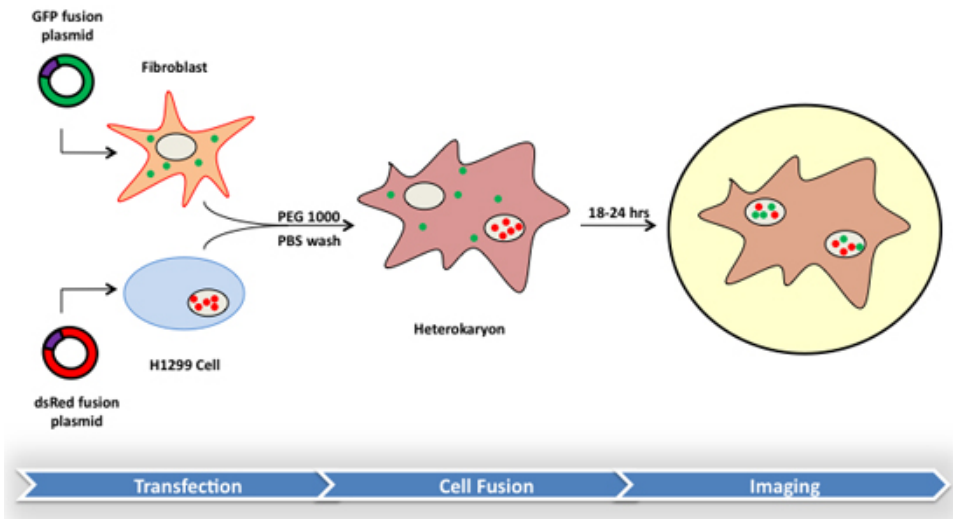
1. Remove culture media from the cells and wash twice with PBS.
2. At this point, it is recommended to treat the cells with cycloheximide (100  $\mu\text{g}/\text{mL}$ ) to inhibit protein synthesis. Allow the cells to incubate for 2 hours and then wash the cells twice with PBS.
3. Fuse cells by exposing them to a solution of 50% polyethylene glycol 1000 (PEG 1000) in serum- free DMEM for 125 seconds at room temperature.
4. Wash cells with PBS to remove the PEG 1000 solution, and add 2 mL freshly warmed and equilibrated media.
5. Allow cells to incubate for 18 - 24 hours.

## Fluorescence Microscopy

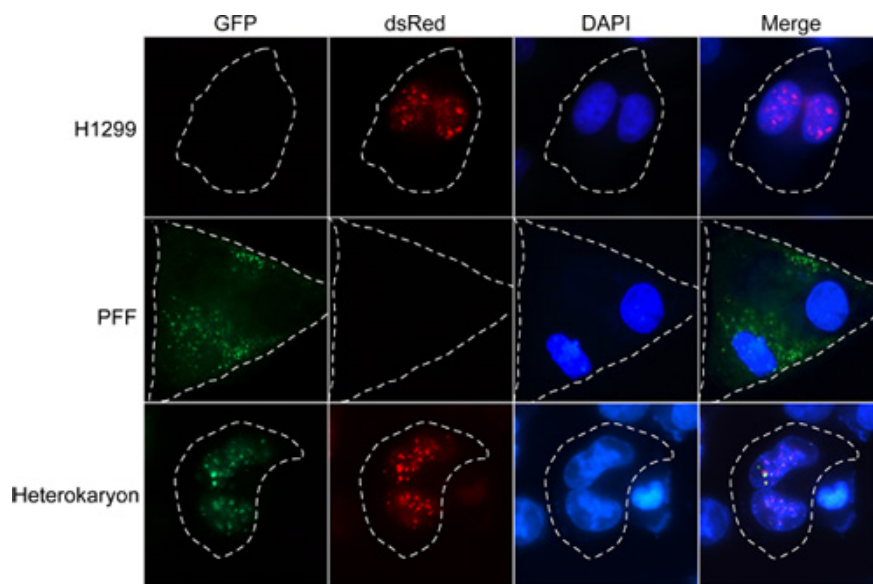
1. Remove the culture media, and wash the cells twice in PBS.
2. Fix cells by adding 1 mL of a 4% paraformaldehyde solution (in PBS) to each well. Gently agitate for 15 minutes.
3. Wash the fixed cells twice in PBS.
4. Carefully remove the cover slips from the plate, wick off excess PBS. Invert onto microscope slides loaded with one drop of mounting medium (90% glycerol, 100mM Tris pH 7.5, 2% DABCO (1,4-diazabicyclo-octane) containing DAPI (4',6-diamidino-2-phenylindole).
5. Remove excess mounting medium from the slides, and seal cover slips with nail polish.
6. Visualize the cells via confocal or epifluorescence microscopy.

## 3. Representative Results

Figure 2 shows an example heterokaryon experiment using primary fibroblasts and H1299 non-small cell lung carcinoma cells. The protein of interest in this experiment (Chicken Anemia Virus-VP3) displays primarily cytoplasmic localization in primary cell types and nuclear localization in transformed cell types as visualized by epifluorescence microscopy. The protein is expressed as a fusion to either GFP (pEGFP-N1 vector, Clontech) in primary foreskin fibroblasts (PFF) or dsRed (dsRed-N1 vector, Clontech) in H1299 cells. Control samples involving self-fusions (top two rows) display multinucleated cells with no change in steady-state localization patterns. Such changes could otherwise occur due to sensitivity to fusion conditions or the formation of syncytia. Heterokaryon fusions (bottom row) demonstrate nuclear entry of the GFP-fused primary cell-derived protein and colocalization with the dsRed-fused protein in response to the introduction of transformed cell material. The example shown is a relatively rare heterokaryon fusion resulting from only two cells, one of each type, as demonstrated by the presence of both fluoroprotein signals. In addition to detecting these types of localization transitions, nucleocytoplasmic shuttling activity can be easily detected by the presence of a single fluorophore in both nuclei. This type of determination is clear when examining 1:1 cell fusions and would depend on inhibition of translation.



**Figure 1. Flow chart of the heterokaryon method using primary fibroblasts and H1299 non-small cell carcinoma cells.** The gene of interest is cloned to produce an in-frame fusion to one of two fluorescent protein genes. Cell lines are then transiently transfected with either construct and allowed to recover. Heterokaryon formation is induced by brief treatment with polyethylene glycol (PEG) followed by further incubation. Finally, the sub-cellular localization of the protein of interest is observed using various forms of fluorescent microscopy.



**Figure 2. Nucleocytoplasmic trafficking and shuttling activity of the Chicken Anemia Virus VP3 protein visualized by heterokaryon fusion.** **Top row:** Representative images of self-fused H1299 cells expressing dsRed-VP3. **Middle row:** Representative epifluorescence images of primary foreskin fibroblast cells (PFF) expressing GFP-VP3 after PEG fusion. **Bottom row:** Heterokaryon fusion of PFF and H1299 cells. Yellow indicates colocalization of GFP and dsRed signals. Cells were imaged using a Leica AF6000E fluorescence microscope and Leica imaging software.

## Discussion

The heterokaryon protocol presented here provides an efficient and easily interpretable method for the characterization of cell type-specific localization behavior as well as nucleocytoplasmic shuttling activity. This method takes advantage of the sensitivity of fluorescence analysis as well as the ability to image live cells and perform studies of protein dynamics. The technique is easily adapted for many different cell types and is amenable to both live and fixed cell imaging. For example, inverted fluorescence microscopy can be used for live imaging and time lapse video capture. By contrast, mounted cells may be used in either epifluorescence microscopy for determination of steady-state localizations or more detailed confocal imaging of discrete localizations. Moreover, techniques such as fluorescence recovery after photobleaching (FRAP), or fluorescence loss in photobleaching (FLIP) can be used in the determination of localization kinetics<sup>1</sup>. The incorporation of alternate fluorophores in the protocol would allow for protein interaction experiments such as fluorescence resonance energy transfer (FRET) to be conducted in concert<sup>2</sup>. Various inhibitors of cellular trafficking such as leptomycin B or dominant negative Ran GTPase constructs can be used to establish dependence on particular import or export pathways<sup>5,6,9</sup>.

- In certain experiments involving shuttling activity, care must be taken to avoid localization effects due to synthesis of new protein. In these cases, translation inhibitors or limited time points must be used. However, artifacts due to translational inhibition remain a possibility. Cell fusions containing one of each cell type are most desired for clear interpretation of shuttling and localization behavior. Optimization of cell number and fusion conditions (timing and concentration) may be necessary to promote such favorable 1:1 fusion events.
- It is important to note that optimization of certain steps in the protocol will be necessary depending on the particular cell types used. Aspects in the experimental procedure such as fusion efficiency, transfection efficiency, and viability after fusion may vary greatly between cell types. In particular, primary cell types can be challenging to manage due to complex culture conditions and low transfection efficiencies. Use of technologies such as the Lonza Nucleofector device can allow for rapid processing of cells as well as dramatically increased transfection efficiencies. Additionally, electroporation methods allow for easy transfer of cells in suspension to the fusion steps that follow.

## Disclosures

No conflicts of interest declared.

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