A non-invasive technique for quantifying and isolating fused cells

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Abstract Cell–cell fusion is an important biological and pathological event. There are limited techniques for studying both the process of cell–cell fusion and the fate of fused cells. We have developed a non-invasive assay for the temporal analysis of cell–cell fusion, quantification of fused cells, and isolation of fused cells. Briefly, cells are transfected with either the T7 bacteriophage RNA polymerase, or yellow fluorescent protein (YFP) driven by a T7 specific promoter. Cells are mixed and induced to fuse. When cells expressing T7 RNA polymerase and T7 promoter driven YFP (T7-YFP) fuse and the cellular contents mix, the YFP is expressed. These YFP-positive cells can be detected with a fluorescent microscope, quantified by flow cytometry, or collected using fluorescence associated cell sorting. Isolated YFP-positive cells can be monitored to

determine the fate of fused cells, specifically for the rates of growth, transformation, and changes in chromosome number.

Keywords Cell–cell fusion · Fluorescence associated cell sorting · Heterokaryons

Introduction

Normal developmental processes, ranging from the fertilization of ovum by sperm to liver cell generation and muscle biogenesis, involve the fusion of two separate cells into one (Chen and Olson 2005). In addition, a common feature of oncogenic viruses is that they mediate cell–cell fusion (Duelli and Lazebnik 2007). While it is debatable as to whether this is the initiating step in oncogenesis, there is increasing evidence that bi-nucleated cells that arise through cell fusion and subsequently divide generate cells that have the chromosomal changes that are characteristic of cancers. These include the gain and loss of chromosomes (aneuploidy) and structural re-arrangement of chromosomes (Duelli et al. 2007). In order to better study both the mechanism by which cells fuse and the fate of fused cells, we have developed a non-invasive assay that monitors the formation of fused cells on a cell-by-cell basis.

We have recently discovered that expression of a known oncogene (HPV16 E5) had fusogenic activity

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(Hu et al. 2009). We first observed that expression of this protein gave rise to bi-nucleated cells. Initially, we could not tell whether these bi-nucleated cells were arising through failed cytokinesis or cell fusion. Our initial attempt to distinguish these two possibilities relied on live cell video microscopy. Even using an automated stage and recording multiple fields simultaneously, performing time-lapse imaging was time-consuming and unpredictable. Since cell–cell fusion occurred in only about 20% of the cells, the probability of imaging cells undergoing fusion was low.

Our second approach for monitoring fusion was to label the nuclei of cells using yellow fluorescent protein (YFP) or red fluorescent protein (RFP) conjugated to histone 2B. Expression of these proteins results in labeling of the nuclei. Following mixing of differentially labeled cells in a 1:1 ratio, cells were treated with the fusogen, and after the appropriate amount of time, the cells were fixed, and each cell was examined by fluorescence microscopy for the presence of heterokaryons—cells with two nuclei of distinct origins. While this protocol is amenable to tracking low probability events, the quantification of heterokaryons by fluorescent microscopy is labor intensive and time consuming.

A previously published assay monitored cell fusion by transfecting separation populations of cells with either T7 RNA polymerase or the β -galactosidase gene driven by a T7 promoter (T7- β -galactosidase) (Kimp-ton and Emerman 1992). β -Galactosidase will be produced when a cell containing T7- β -galactosidase fuses with a cell producing T7 RNA polymerase. The relative amount of cell fusion can be determined by measuring β -galactosidase activity using standard colorimetric assays on fixed cells or cell lysates. Since T7 RNA polymerase is normally only expressed in bacteriophage, the basal level of T7- β -galactosidase transcription is negligible. Similar assays have been reported using luciferase as a reporter gene (Sakamoto et al. 2003). However, we did not favor these techniques because they are invasive—cells need to be fixed or harvested to determine if fusion occurred. This technique does not allow investigators to know if cell fusion has occurred until they are committed to the assay. If the time frame for studying fusion were incorrect, the experiment would need to be restarted de novo.

To overcome these obstacles, we modified this reporter assay by substituting the β -galactosidase gene with YFP (Fig. 1). This modification offers a number

Fig. 1 Schematic of cell fusion assay. Cells are transfected with cDNA plasmids encoding either T7-RNA polymerase (driven by a cytomegalavirus promoter) or yellow fluorescent protein (YFP) driven by a T7-promoter. Transfected cells are mixed in a 1:1 ratio and fusogen is added. At various times, fused cells are monitored by fluorescence microscopy, or analyzed by FACS to quantify the number of fused cells or to isolate fused cells

of advantages. First, living cells can be examined with a fluorescence microscope for the presence of fused (YFP-positive) cells. Once the frequency of cell fusion is noted, the cells can be returned to growth conditions, and monitored again at later time points. Second, cells can be harvested, and analyzed by flow cytometry for the presence of fused cells. Cell sorters can examine thousands of cells per second, which allows for the rapid processing of samples on a cell-by-cell basis. Finally, fluorescence associated cell sorting allows the user to collect those cells that have undergone fusion. With proper instrumentation, individual cells can be isolated for clonal analysis or fused cells can be collected for biochemical analysis. These three features of our modified assay have made this a more versatile assay that expands our opportunities for studying both the mechanism of cell fusion and the consequence.

Materials and methods

Dulbecco's minimal essential media (DMEM), G418 sulfate, streptomycin and penicillin were obtained

from Invitrogen (Carlsbad, CA). The TE2000 inverted fluorescent microscope was purchased from Nikon (Melville, NY). The BD FACSCalibur and BD Influx Cell Sorter were obtained from BD Biosciences (San Jose, CA).

Procedures

Plasmids and cloning

pRK7-T7 RNA polymerase was generated by using PCR primers to clone T7 RNA polymerase from BL21 *E. coli* (primer pairs 5' TTA CAT TCT AGA ATG AAC ACG ATT AAC ATC GCT AAG- 3' and 5' ATG TAA CTC GAG TTA CGC GAA CGC GAA CGC GAA GTC CGA-3'). The primers incorporated XbaI and XhoI restriction enzyme sites. The resulting PCR product was digested with XbaI and XhoI, and ligated into the same sites in pRK7 (with a multiple cloning site that has been altered to include an XhoI site). The resulting plasmid is such that the gene expression was driven by the plasmid's CMV promoter. The T7 promoter driving the expression of His6-S-tagged YFP in pET30 (Novagen) was amplified using PCR primers that added SpeI sites upstream of the T7 promoter and downstream of the T7 terminator. The PCR product was digested with SpeI, and subcloned into the pRK7 that was digested with SpeI and XbaI to remove the CMV promoter. The resulting plasmid is referred to as pT7-YFP.

Tissue culture and transfection

tTA-HeLa cells are a clonal cell line that has been stably transfected with the tetracycline transactivator (tTA) (Gossen and Bujard 1992). Cells were a kind gift of Dr. Sandra L. Schmid for the Scripps Research Institute and have been described previously (Damke et al. 1994). Cells were maintained in DMEM supplemented with 5% fetal bovine serum, 100 units/mL penicillin, 100 µg/mL streptomycin, and 2 mM glutamine at 37 °C in 5% CO₂. Cells were grown in 400 µg/mL G418 to select for transfection with the tTA.

tTA-HeLa cells (2×10^6) were transfected with 10 µg of either plasmid by calcium phosphate transfection. Cells were allowed to recover from transfection for 24 h in growth media. In control cells

transfected in parallel, transfection efficiency of a plasmid containing a YFP-tagged fusion protein (pRK7-YFP-histone 2B) was estimated to be >90% 72 h after transfection (~80% efficiency 48 h after transfection). Following recovery, cells were collected and pRK-T7 RNA polymerase and pT7-YFP transfected cells were mixed in a 1:1 ratio. Cells were plated at ~90% confluence. After re-plating (24 h), cells were either transduced with a tetracycline-regulatable (tet-off) adenovirus encoding HPV16 E5 (a fusogenic protein) (Hu et al. 2009) or treated with polyethylene glycol for 5 min (Madan and DeFranco 1993). Adenovirally infected cells were transduced at a multiplicity of infection (m.o.i.) of ~20 plaque forming units (pfu)/cell. Under these conditions, >90% of cells were positive for HPV16 HA-E5 based on indirect immunofluorescent staining with the anti-HA antibody. The addition of 1 µg/mL tetracycline to adenovirally infected cells abrogates expression of HPV16 E5.

Visual monitoring cell fusion

Live cell images were monitored for the presence of fluorescent cells using a Nikon TE2000 inverted fluorescent microscope, captured with an ORCA AG12 CCD camera, and processed with Openlab software. Images of fixed cells were captured using an Olympus AX70 epifluorescent microscope with Q-capture software.

Flow cytometry

Cells were harvested the same way for quantitation and isolation of fused cells. At various times after the induction of fusion, cells were washed two times with room temperature phosphate buffered saline (pH 7.3) at room temperature, trypsinized until cells came loose from the plate, and resuspended in growth media. Cells were washed by pelleting the intact cells with a low speed centrifugation ($200 \times g$), using a IEC Centron GP8R centrifuge. Following centrifugation, the supernatant was discarded, and the resulting cells were resuspended to 10^6 cells/mL. Cells were analyzed by either flow cytometry using a BD FACSCalibur™ (BD Bioscience, San Jose, CA) or cell sorting using a BD Influx Cell Sorter (BD Biosciences, San Jose, CA).

The relative change in cell–cell fusion (i.e., in the absence or presence of a fusogen), can be monitored by

comparing the percentage of YFP positive cells or by comparing the Total Fluorescence Intensity (calculated by multiplying the mean fluorescence intensity by the percentage of YFP-positive cells) which accounts for the level of fluorescence in the entire cell population (Kimpton and Emerman 1992; Nussbaum et al. 1994).

Immunoblot

Cell lysates and immunoblotting

Cell lysates were prepared as previously described (Dinneen and Ceresa 2004). Total cell lysates (30 µg) were resolved on 16% Tris-Tricine gels and transferred to nitrocellulose. Membranes were immunoblotted using a mouse monoclonal anti-HA (12CA5) antibody (Roche) followed by probing with a horseradish peroxidase-conjugated goat anti-mouse (Pierce). Proteins were visualized with enhanced chemiluminescence and documented using a UV products imaging system.

Results and discussion

Shown in Fig. 2 is an image of live cells that have been induced to undergo cell fusion. Images were collected at 48-h after treatment with the fusogen (HPV16 E5). YFP-positive cells can easily be observed through live cell imaging (Fig. 2a). To demonstrate that the YFP-positive cells are bi-nucleated, the cells were fixed, subjected to indirect immunofluorescence (anti-GFP), and DAPI staining (Fig. 2b). It has been our experience, using the proteins that we study, that the production of YFP peaks around 72 h after induction. This may be slower than the kinetics of cell fusion, as additional time is required for the transcription, translation, and oxidation of the YFP.

It is important to note that this protocol measures a fraction of the cells that are undergoing cell–cell fusion—those that produce YFP when their cytosols mix. If two pRK7-T7 RNA polymerase-transfected cells fuse, no fluorescence will be produced. The same is true if two pT7-YFP cells fuse. For a given experiment, one could establish a ratio of YFP-positive cells to bi-nucleated cells and use the

Fig. 2 Visual analysis of cell–cell fusion in HeLa cells. HeLa cells were transfected with T7-RNA polymerase and T7-YFP as described in “Materials and methods” and infected with an adenovirus to express a fusogenic protein (HPV16 E5). **a** After 48 h, live cells were examined by fluorescent microscopy for the presence of GFP-positive cells. Images were collected using a Nikon TE-2000 fluorescent microscope (20× objective) and processed using OpenLab Imaging software. Size bar = 10 µm. **b** Cells treated as described in **a** were fixed and subjected to indirect immunofluorescence using an anti-GFP antibody (to enhance the lower signal observed in permeabilized cells) and stained with DAPI (Dinneen and Ceresa 2004). Images were collected using an Olympus AX70 epifluorescent microscope (40× objective) and Q-Capture software. Size bar 10 µm

detected YFP-positive cells to estimate the total number of cell–cell fusion events.

Fused cells can be quantified by flow cytometry. Representative scattergrams (Fig. 3a) provide a visual representation of the efficiency of cell fusion. Since there is typically a basal level of fluorescence associated with cells (ranging from 0.1 to 1.0% of cells), data are normalized to that value. Data can be plotted as the change in the percentage of fluorescent cells (Fig. 3b) or total fluorescence activity (percentage of fluorescent cells times the mean fluorescent

activity; Fig. 3c). Total fluorescence activity accounts for fluorescence of the entire population of cells. This readout is consistent with the data presentation from experiments using the β -galactosidase reporter (Kimpton and Emerman 1992; Nussbaum et al. 1994). The inset in Fig. 3b shows a Western blot of a fraction

Fig. 3 Quantitative analysis of cell–cell fusion in HeLa cells. Cells were transfected as described in “Materials and methods”, and infected with a tetracycline-regulatable (“tet-off”) adenovirus that causes cell fusion (HPV16 E5). As controls for infection, cells were either infected with nothing (“No Virus”), virus in the presence of 1 μ g/mL tetracycline (“E5 + tet”), or an adenovirus encoding β -galactosidase (β -gal) to control for any potentially deleterious adenoviral effects. After recovery (72 h), cells were analyzed using a BD FACSCalibur. **a** Representative Flow cytometry data from each experimental condition. Shown are scattergrams for each of the four cell populations. Each dot on the graph represents a single cell and its associated YFP fluorescence (FL1-H) and Red fluorescence (FL2-H). Non-specific auto-fluorescence activity is characterized by a proportional increase in FL1-H and FL2-H. Only cells with specific increases in YFP activity were scored as positive (boxed region). The percentage of positive cells is indicated within the boxed area. **b** Data plotted as the percentage of fluorescent bi-nucleated cells. Inset immunoblot using the anti-HA antibody (12CA5) to demonstrate the expression of HPV16 HA-E5. **c** Data plotted as the total fluorescence (mean fluorescent intensity $\times$ percentage of positive cells). This measurement takes into account the fluorescence associated with the entire population of cells. * Indicates p value <0.01 as compared any of the three control conditions

of the cells that were analyzed by FACS to show that the fusogen (HPV16 HA-E5) was expressed.

This protocol allows for the efficient isolation of fused cells using fluorescence associated cell sorting. By enriching those cells that have undergone fusion, one can easily monitor the fate of such cells. This is particularly of interest to those cancer researchers that hypothesize that cell fusion precedes the development of aneuploidy in some cancers (Duelli and Lazebnik 2007; Parris 2008a, b; Pawelek and Chakraborty 2008a, b). Using this technique, experiments could be performed to isolate fused cells and monitor cell viability, transformation by colony formation assay, or chromosome number and structure by spectral karyotype analysis.

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