Chapter 17

Immunodetection of Human LINE-1 Expression in Cultured Cells and Human Tissues

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Abstract

Long interspersed element-1 (LINE-1) is the only active protein-coding retrotransposon in humans. It is not expressed in somatic tissue but is aberrantly expressed in a wide variety of human cancers. ORF1p protein is the most robust indicator of LINE-1 expression; the protein accumulates in large quantities in cellular cytoplasm. Recently, monoclonal antibodies have allowed more complete characterizations of ORF1p expression and indicated potential for developing ORF1p as a clinical biomarker. Here, we describe a mouse monoclonal antibody specific for human LINE-1 ORF1p and its application in immunofluorescence and immunohistochemistry of both cells and human tissues. We also describe detection of tagged LINE-1 ORF2p via immunofluorescence. These general methods may be readily adapted to use with many other proteins and antibodies.

Key words LINE-1 ORF1p, p40, Immunofluorescence, Immunohistochemistry, Tissue microarray, Tumor marker, Biomarker

1 Introduction

Long interspersed element-1 (LINE-1) activity is a significant determinant of our genome sequence. LINE-1 and other sequences mobilized by LINE-1-encoded proteins together constitute about 35 % of our DNA [1]. Ongoing LINE-1-mediated retrotransposition makes these sequences important sources of genetic structural variation in humans as well as a source of instability in cancer genomes [2–6].

LINE-1 is the only active protein-coding family of transposable elements in humans. The first of its two open reading frames encodes ORF1p, a 40 kDa protein with RNA-binding activities required for LINE-1 retrotransposition [7–9]. The second, ORF2p, encodes the endonuclease and reverse transcriptase activities which are also essential for LINE-1 propagation [10, 11] and is expressed at extremely low levels [12].

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ORF1p comprises an N-terminal domain, a coiled-coil domain (CCD), an RNA recognition motif (RRM), and a C-terminal domain. The protein associates with LINE-1 ribonucleoprotein (RNP) complexes as a trimer brought together through CCD interactions [13–15], and two to ten trimers associate with each LINE-1 RNP [16]. LINE-1 ORF1p is an abundant protein in cells expressing LINE-1. It can be readily seen on Coomassie gels of total protein when cells are transfected with LINE-1-expressing constructs under the native promoter; it may be a dominant band using CMV or CAG promoters. In terms of total protein expression, ratios of ORF1p:ORF2p are in the range of 1000–10,000:1. Using immunoprecipitation and two distinct quantitative staining methods as well as quantitative mass spectrometry, the ratio of ORF1p:ORF2p in RNPs in these cells is between 6:1 and 30:1. Together, these data show that a substantial portion of ORF1p is not engaged in the LINE-1 RNP [16].

To develop a mouse monoclonal antibody to detect LINE-1, 15 peptides from the human LINE-1 ORF1p sequence (AAB60344.1, 338 AA) were selected for immunization of BALB/c mice. The antibody referenced in this chapter recognizes amino acids 35–44 (MENDFDELRE) of ORF1p [17]. This is a region of the protein where mouse and human LINE-1 sequences diverge; it precedes the CCD and has not been solved in protein crystallography studies [15]. Using immunohistochemistry, we found nearly half of human cancers stain positively for ORF1p, with immunoreactivity in some common cancers approaching 100 % of cases. No staining was observed in the cognate normal tissues [17].

2 Materials

2.1 For Cell Culture IF

1. Cover slips, size 22 × 22 × 1.
2. Slides, size 25 × 75 × 1.
3. Forceps: Electron Microscopy Services Style 4A.
4. Parafilm M: 4″ width.
5. Fibronectin from human plasma.
6. Poly-L-lysine.
7. Hoechst 33342 trihydrochloride, trihydrate: 10 mg/mL Solution in water.
8. Bovine serum albumin (BSA), high avidity, 30 % solution.
9. *PBS/glycine*: We make standard 10× PBS and a 100× glycine-azide stock. Sodium-only or sodium-potassium PBS recipes may be used.

10. *100× Glycine*: 1 M glycine plus 2 % sodium azide, adjust pH to 7.4 with NaOH or HCl. This is stable at room temperature for months to years.
11. *Mounting media:* 0.1 M *N*-propyl-gallate in glycerol. Add *N*-propyl-gallate. Prepare and dissolve at 65 °C overnight. Stable at room temperature for months to years.
12. *Fixative*: 3 % paraformaldehyde (PFA) in PBS (makes 100 mL) (*see* **Note 1**).
    (a) Weigh 3 g PFA, and then add 80 mL ddH_2O and 250 μL 1 N NaOH.
    (b) Heat at 50 °C in a water bath to dissolve completely.
    (c) Add 10 mL 10× PBS and adjust volume to 100 mL with ddH_2O.
    (d) Check pH, and adjust to 7.4 with NaOH or HCl if needed.

2.2 For Tissue IHC and IF

1. Xylene.
2. Absolute ethanol.
3. TBS: 50 mM Tris–HCl, pH 7.4, 150 mM NaCl.
4. TBST: TBS plus 0.05 % (v/v) Tween 20.
5. BSA.
6. BSA-TBST: 1 % BSA (w/v) dissolved in TBST.
7. Citrate antigen retrieval buffer: 10 mM Sodium citrate, pH 6.0, 0.05 % Tween 20.
8. EDTA antigen retrieval buffer: 1 mM EDTA, pH 8.0, 0.05 % Tween 20.
9. 3 % (v/v) Hydrogen peroxide in dH_2O.
10. For signal amplification (option one): Histostain-SP Kit Broad Spectrum Kit (Life Technologies).
11. For signal amplification (option two): EnVision HRP Mouse (DAB+) or Rabbit (DAB+).
12. For HRP-catalyzed amplified immunofluorescence using tyramide: Perkin Elmer™ TSA Plus Fluorescein or Cy-5.

2.3 Antibodies

Mouse anti-LINE-1 ORF1p clone 4H1 (10 mg/mL), stored in 50 % glycerol at –20 °C.

Rabbit anti-LINE-1 ORF1p clone JH73 (gift of Jeff Han).

Alexa 488 GOAT Anti-Mouse.

Alexa 568 GOAT Anti-Rabbit.

Rabbit Anti-GFP polyclonal antibody.

Rabbit Anti-Flag polyclonal antibody.

Mouse Anti-Flag M2 (*see* **Note 2**).

3 Methods

3.1 Immunofluorescence (IF) Detection of LINE-1 ORF1p and ORF2p from Human Cell Lines

Immunofluorescent staining (IF) for LINE-1 allows sensitive detection of LINE-1 expression and demonstrates subcellular co-localization of both ORF proteins. LINE-1 proteins accumulate in the cytoplasm and are relatively excluded from cell nuclei. For reasons that are not understood, we find that expression of tagged ORF2p in HEK-293T and HeLa cell lines varies greatly from cell to cell in a seemingly stochastic manner [16]. IF was performed in transiently transfected or puromycin-selected HeLa and HEK-293T cells grown on glass cover slips using both constitutive and tetracycline-inducible promoters, but this standard IF protocol works well with many cultured cell lines and antigens. Many IF protocols use hundreds of μL of antibody solution per sample and often take days to complete. With this protocol [18], slides can be visualized in less than 2 h and, by using small droplets (50 μL) of antibody on Parafilm M, it saves limited and costly reagents. For ORF1p, untagged protein can be visualized using either of the two recently developed monoclonal antibodies, which were verified in comparison with Flag-tagged ORF1p. 3× Flag-tagged and GFP-tagged ORF2p can be detected readily; robust immunodetection of untagged ORF2p is currently a technical barrier in the field. Example images are shown in Fig. 1.

Many variations on the protocol below are effective. Here, we present our standard protocol for transient transfection, along with some options for customization.

3.1.1 Timeline

Day 1: Plating and Transfection

9 a.m.: Plating

5 p.m.: Transfection

Day 2: Permeabilization, Fixation, and Immunostaining

Considerations: For optimal results, plating should occur >8 h before transfection. For maximum transfection efficiency, cells can be plated 16–24 h beforehand, but because this results in fewer single cells under the scope, we prefer using the 8-h interval described. After 3 days on glass, cultured cells are often very clumpy and their borders may be difficult to distinguish. Therefore, for staining more than 18–24 h after transfection, plating on cover slips can be done after transfection. ORF2p expression peaks at 20–24 h post-transfection/induction and can be very difficult to detect at <16 h.

3.1.2 Protocol

Day 1: Plating and Transfection

1. *Plating considerations*

   For strongly adherent cells such as HeLa, uncoated glass cover slips can be used. For weakly adherent cells such as 293T or non-adherent hematopoietic lines, cover slips should be

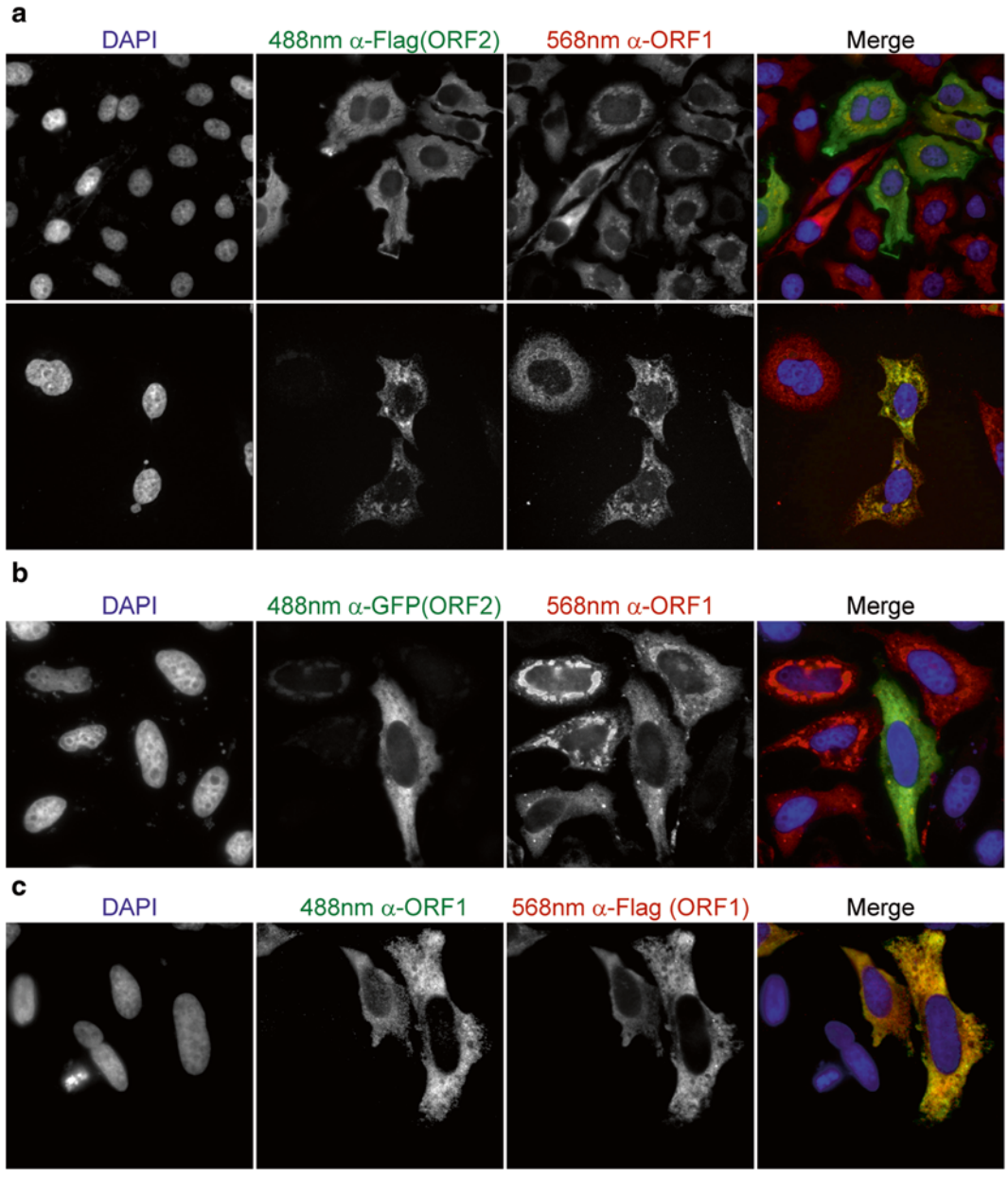

Fig. 1 Example immunofluorescent staining of LINE-1. (**a**) Staining of HeLa cells expressing pLD401 (untagged ORF1, ORF2-3×Flag) using mouse anti-Flag and rabbit anti-ORF1 JH73. *Top row*: epifluorescence, 40×. *Bottom row*, confocal, 40×. (**b**) Staining of HeLa cells expressing pLD402 (untagged ORF1, ORF2-GFP) using rabbit anti-GFP and mouse anti-ORF1 4H1; epifluorescence, 64×. (**c**) Control staining of HeLa cells expressing pLD288 (ORF1-Flag, untagged ORF2) with rabbit anti-flag and mouse anti-ORF1 4H1; epifluorescence, 64×

coated with a substrate to promote attachment of the cells. Fibronectin glycoprotein or poly-L-lysine, a positively charged synthetic amino acid chain, is widely used for this purpose. We autoclave cover slips in glass petri dishes; cover slips stuck together after autoclaving are discarded.

2. *Coating cover slips with fibronectin.*
   1. Fibronectin from human plasma is resuspended in sterile phosphate-buffered saline (PBS) and stored in 60 µL aliquots at −80 °C.
   2. Dilute a 60 µL aliquot 1:100 to 6 mL in sterile PBS (10 µg/mL final).
   3. With cover slips in 6-well wells or 35 mm dishes, add 1 mL to each cover slip, leaving it beaded up on top of the glass.
   4. Incubate for >2 h at room temperature in a tissue culture hood. Alternatively, >1 h at 37 °C, or overnight at 4 °C. For overnight incubation, seal with Parafilm.
   5. When ready to use, aspirate and wash with PBS or media.
3. *Coating cover slips with poly-L-lysine*

   Poly-L-lysine is less expensive than fibronectin and works well with HEK-293T cells.
   1. Poly-L-lysine (mol wt ≥300,000) is dissolved to 10 mg/mL in sterile ddH_2O and 400 µL aliquots are stored at −20 °C.
   2. Make 4 mL of a 1 mg/mL working dilution in ddH_2O (400 µL stock + 3.6 mL ddH_2O).
   3. With cover slips in 35 mm dishes or 6-well plates, apply the solution to each cover slip for ~30 s to 1 min.
   4. Pipet to sequential dishes/wells, coating ~5–10 per 1 mL solution. We routinely coat up to 40 cover slips with 4 mL.
   5. Wash each coated cover slip with 2× 1 mL ddH_2O, aspirating off all of the solution.
   6. After the second wash, aspirate all the water off and allow to air-dry completely in the hood (*see* **Note 3**).
   7. Store at room temperature for weeks to months before use, or use once dry (minimum ~30 min).
4. *Plating*

   Cultured cells can be subconfluent or confluent, but not overgrown.
   1. Insert an autoclaved cover slip or coated cover slip into each 35 mm dish or 6-well plate, discarding cover slips that are clumped together.
   2. If plating HEK-293T the day before staining as in the above timeline, plate 300,000–400,000 cells. Plate 200,000 if plating the day before transfection (2 days on the plates). If

cells are to be on the plates longer than 2 days, plate fewer cells.

5. *Transfection*

We prefer Fugene 6 or HD (Promega) over Lipofectamine (Life Technologies) and XtremeGene (Roche) reagents in HEK-293T and HeLa due to reduced toxicity and better reproducibility. Transfection is done following the manufacturer's standard protocol with a 3 μL:1 μg ratio of reagent:DNA. DNA should be midi- or maxi-prepped; minipreps are not suitable.

Day 2: Fixation, Permeabilization, and Staining (*See* **Note 4**)

1. *Paraformaldehyde (PFA) Fixation and Triton Permeabilization*

This is a standard fixation method for immobilizing antigens. Paraformaldehyde acts as a cross-linking reagent and is followed by permeabilization with Triton X-100 detergent. After PFA fixation, glycine is added to the buffers as both a quench and blocking agent. Saponin detergent is also popular and may expose different epitopes. A methanol treatment at −20 °C can be used as an alternative to cross-linking fixation and permeabilization. Organic solvent fixations remove lipids and dehydrate cells and may provide better access to antigens or better preservation of some organelles such as the endoplasmic reticulum.

1. Thaw an aliquot of 3 % PFA solution.
2. Make fresh working stock of 0.5 % Triton X-100 in PBS/glycine from 10 % stock (at least 1 mL per sample).
3. Aspirate media, and wash cells with PBS (without glycine).
4. Aspirate, wash, and fix cells: Add 1 mL 3 % PFA in PBS for 10 min.
5. Aspirate PFA, and wash with PBS/glycine.
6. Aspirate, wash: Permeabilize cells by adding 1 mL fresh 0.5 % Triton X-100 in PBS/glycine. Incubate for 3 min at room temperature.
7. Rinse in PBS/glycine, and let sit in PBS/glycine for at least 5 min to fully quench (or longer).

2. *Immunostaining*

To minimize the volume of antibody solution used, we take advantage of the hydrophobicity of Parafilm M, applying the cover slip cell-side down onto a beaded drop of antibody solution. The cell side of the cover slip is coated in the antibody solution via surface tension. The minimum volume required for this using a standard 22 mm cover slip is approximately 35 μL; the extra volume reduces edge drying and makes handling the cover slips easier. All antibodies are diluted in *PBS/glycine/1 % bovine serum albumin (BSA)*; BSA serves as both diluent and blocking agent.

Multichannel fluorescent staining and the species problem. Using standard UV-fluorescent microscopes and fluorophores, up to four orthogonal channels may be studied simultaneously; our common filter set is blue/DAPI (excite: 360 nm, emit: ~460 nm), green/FITC (excite: 488 nm, emit: ~525 nm), red/Texas Red (excite: 568 nm, emit: ~615 nm), and far-red/Cy5 (excite: 633 nm, emit: ~680 nm) (*see* **Note 5**). Thus, in principle, up to four different targets may be visualized simultaneously using immunofluorescence. In practice, the ultraviolet channel is usually reserved for DNA dyes (DAPI, Hoechst), and detecting three different targets requires primary antibodies produced in three different species with a panel of orthogonally reactive fluorescent-labeled secondary antibodies. In selecting the panel of secondary antibodies, using three antibodies raised in the same species can reduce background. Further, using highly cross-adsorbed secondary antibodies can eliminate cross-binding to off-target primary antibodies. Note that in the first use of any secondary antibody combination, it is important to include "no-primary" controls for each primary antibody individually (*see* **Note 6**).

For LINE-1, a typical staining is a rabbit anti-ORF1p monoclonal JH73 (0.25 μg/mL) and mouse anti-Flag M2 (2 μg/mL, ORF2p-3×Flag) along with Hoechst stain for DNA, and might include chicken anti-actin. In our hands, the signal-to-noise ratio tends to be better in the green (488 nm) channel, so we usually stain for ORF2p with this secondary antibody. Alternatively, mouse anti-ORF1 4H1 (1 μg/mL) can be used with rabbit polyclonal anti-flag or many other commercial DYKDDDK reagents; however the 3×Flag signal is weaker in this case because the 3×Flag tag is optimized for binding the M2 antibody.

1. Dilute primary and secondary antibodies. Note: primary antibody dilutions should be optimized. First make a solution of PBS/glycine/1 % BSA using the 30 % BSA stock (*see* **Note 7**).
2. Stretch and label Parafilm on the bench as in Fig. 2. Label each square with both the cover slip number and which stain that square receives.
3. Add 50 μL of the primary antibody mix to the center of the corresponding squares on the Parafilm. Avoid bubbling the drop.
4. Using a pair of sharp forceps, carefully remove the cover slip from the dish. (This is much easier with buffer present.) Dry the cell side of the cover slip by blotting and flip it *cell-side down* onto the antibody drop (*see* Fig. 3). *Incubate for 20 min at room temperature.*
   (a) Set a stack of two paper towels on the bench for blotting.

Fig. 2 Antibody droplets on Parafilm M. For each cover slip, one square of 4″ wide Parafilm M is used. Label each square with the antibody combination to be used. Pipet a 50 μL droplet of primary or secondary antibody, and then apply the cover slip *cell-side down* for 20 min at room temp. Note that 30–35 μL is the minimum needed, but higher volume increases consistency and ease of handling. Avoid bubbling the drop; remove bubbles with a pipet

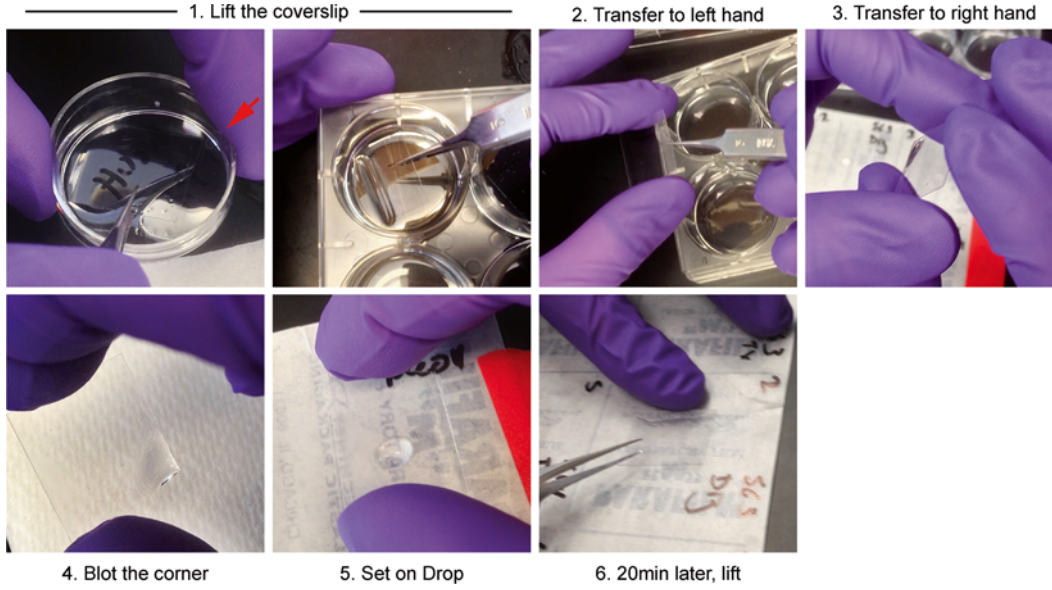

Fig. 3 Transferring the cover slip from dish or plate to droplets on Parafilm. Lift the cover slip from buffer-containing dish/plate with forceps (*1*). With dishes, pinching the dish firmly (*red arrows*) helps. Wear gloves. To avoid breaking or dropping the cover slip, transfer the cover slip carefully from the forceps to the left hand (*2*), then back to the right hand (*3*) as shown. Tilt the cover slip so that a drop forms in the corner (*4*) and blot the bulk off using a small stack of paper towels, touching only the edges of the slide to the paper towel. Set the blotted cover slip *cell-side down* on the drop (*5*). If any bubble forms, gently chase it out by pushing on the back or lift the cover slip and remove. Incubate for 20 min at room temperature. To lift cover slip, use a finger and gentle pressure as shown to keep the cover slip in place (*6*)

(b) Lift the cover slip with fine forceps (*see* **Note 8**).

(c) Very coordinated scientists can use forceps to flip, blot, and transfer the cover slip to the drop. For the rest of us, we recommend the following method (right-handedness is presumed):

- Lift the cover slip with forceps and transfer the cover slip into your left hand, holding it gently by the edges and paying careful attention to which side is the cell side. Set the forceps down and transfer the cover slip back to your right hand, grasping the free edges.
- Angle the cover slip downwards, cell-side down. A drop of liquid will accumulate in the corner. Gently blot the edge on the paper towel, and then set the cover slip cell-side down down on the antibody droplet.
- Notes:

  During this step it is easy to make a mistake and shatter the cover slip by dropping it or lose track of which side has the cells. Work carefully and focus; *it gets easier with practice.*

  If the cover slip falls and you are not sure which side the cells are on, the best way to check is to lift the slip and carefully scrape a corner. The cell side scrapes clean; nothing happens on the other side.

5. During the incubation, aspirate the PBS/glycine out of the dishes and replace with fresh buffer for the first wash.
6. Using forceps, carefully lift the cover slip off the Parafilm and flip it back CELL-SIDE UP into the dish (containing fresh PBS/glycine wash).
   (a) You can keep the cover slip from sliding away by putting a fingernail on the opposite side (*see* **Note 9**).
7. Shake gently to wash, and aspirate off the wash.
8. Wash again with PBS/glycine.
9. Clean the Parafilm with a paper towel and rinse, dry, and repeat for two washes total. (Alternatively, replace the Parafilm with a clean sheet.)
10. Stain with secondary antibody: Repeat **steps 2**–**7** using the secondary antibody, but modify the wash procedure as below to include Hoechst stain in the first wash.
    (a) During the secondary antibody incubation, make a working stock of Hoechst DNA Dye by diluting the 10 mg/mL stock 1:100,000 total in PBS/glycine in sequential 1:100 and then 1:1000 dilutions (final 100 ng/mL).

(b) Add 1 mL to each well for the first wash. Once the cover slips are added, incubate for 5 min at room temperature.

(c) Aspirate and wash with PBS/glycine. Let sit in PBS/glycine until ready to mount.

3. *Mounting*: Many different mounting media are available, and there are pros and cons to each. We typically use a simple and inexpensive mounting media using 0.1 M *N*-propyl gallate in glycerol [19]. Slides are stable for weeks to months if stored flat, and can be viewed on the scope the instant they are mounted. They can also be un-mounted easily if needed (*see* **Note 10**). Some mounting media also contain DAPI stain. We prefer not to seal cover slips with clear nail polish as the raised surface can damage microscope objectives, although this can be helpful for preserving slides with liquid mounting media over time.

   1. Pre-label the slides, and clean with Kimwipes and/or a compressed air can. Each slide should be marked with sample name, date, permeabilization, and stain, as appropriate. If two samples are to be compared directly, it is helpful to put both cover slips on the same slide.
   2. To mount each cover slip, we follow a protocol analogous to the staining procedure, followed by aspiration of excess mounting media.

      (a) Put a drop of mounting media for each cover slip on the slide. Remove the cover slip from the dish, blot to remove buffer from the cell side, and then flip cell-side down onto the drop. It may be helpful to dry the back of the cover slip before mounting.

      (b) Apply gentle downward pressure on each cover slip with index and middle fingers and a cut pipet-tip aspirator while aspirating excess mounting media from around the edges of the cover slip. Then, maintain firm downward pressure with your fingers. Push downward and sideways with the plastic pipet tip against the top of the glass cover slip, chase out large bubbles, and continue to remove excess mounting media accumulating at the edges of the glass. Push firmly enough to move air bubbles to the edge but not excessively, as sliding cover slips or excessive pressure can shear the cells.

      (c) For those inclined to remove *every single drop* of excess mounting media, allow the slides to sit for a few minutes and then make a second pass over the completed slides.

3.2 Immunohistochemical and Immunofluorescent (IF) Detection of LINE-1 ORF1p from Human Tissues

This protocol describes immunolabeling of formalin-fixed, paraffin-embedded (FFPE) tissue sections using the monoclonal antibody for LINE-1 ORF1p. This is a general staining protocol we have used in human pancreatic tissue, but it can be modified to accommodate staining of other tissues and cell lines. Two alternative approaches for detection are described here: immunohistochemistry (IHC), where the protein of choice is detected via binding of a chromogen such as 3,3′-diaminobenzidine (DAB) and visualized using a light microscope; or immunofluorescence (IF), where the protein of choice is detected via binding of fluorophores and visualized using a fluorescence microscope. Alternatives to DAB for immunohistochemistry include chromogens such as 3-amino-9-ethylcarbazole (AEC). Both the IHC and IF protocols described herein have been optimized and validated with proprietary signal amplification technologies as well as fluorescent-conjugated secondary antibodies. We routinely use 10 % neutral buffered formalin for tissue fixation, although B5, Bouin's, zinc formalin, or alcohol-based fixatives are also commonly used.

For IHC, in [17] we used Invitrogen's Histostain-Streptavidin-Peroxidase (SP) Broad Spectrum Kit for LINE-1 ORF1p detection, with a protocol modified from that provided by the manufacturer. The kit uses a biotinylated secondary antibody and horseradish peroxidase-labeled streptavidin for signal amplification. This complex is then visualized through the use of a DAB chromogen mixture and results in highly specific signal for LINE1 ORF1p. Example IHC images of LINE-1 ORF1p using the 4H1 monoclonal antibody are shown in Fig. 4.

As an alternative to the Histostain kit, we use the EnVision System for signal amplification. EnVision consists of a poly-horseradish peroxidase (HRP) chain secondary antibody. In our experience, this system gives more robust signal over background than the Histostain kit.

For immunofluorescence, detection can be achieved directly with fluorescent-conjugated secondary antibodies; we prefer Alexa-fluor-labeled antibodies raised in goat as described in cell culture immunofluorescence above (*see* **Note 11**).

A common protocol follows for deparaffinization through primary antibody incubation. The protocol splits at this point for IHC and IF.

3.2.1 Protocol

1. *Deparaffinization and rehydration*:
   1. Label slides with a pencil (*see* **Note 12**).
   2. Bake slides at 60 °C for 20–30 min to melt paraffin.
   3. Perform the following series of washes to remove paraffin:
      (a) Xylene washes for 5 min, 5 min, and 3 min (using the fume hood).
      (b) Wash in 100 % ethanol for 2 min. Repeat twice for a total of three washes.

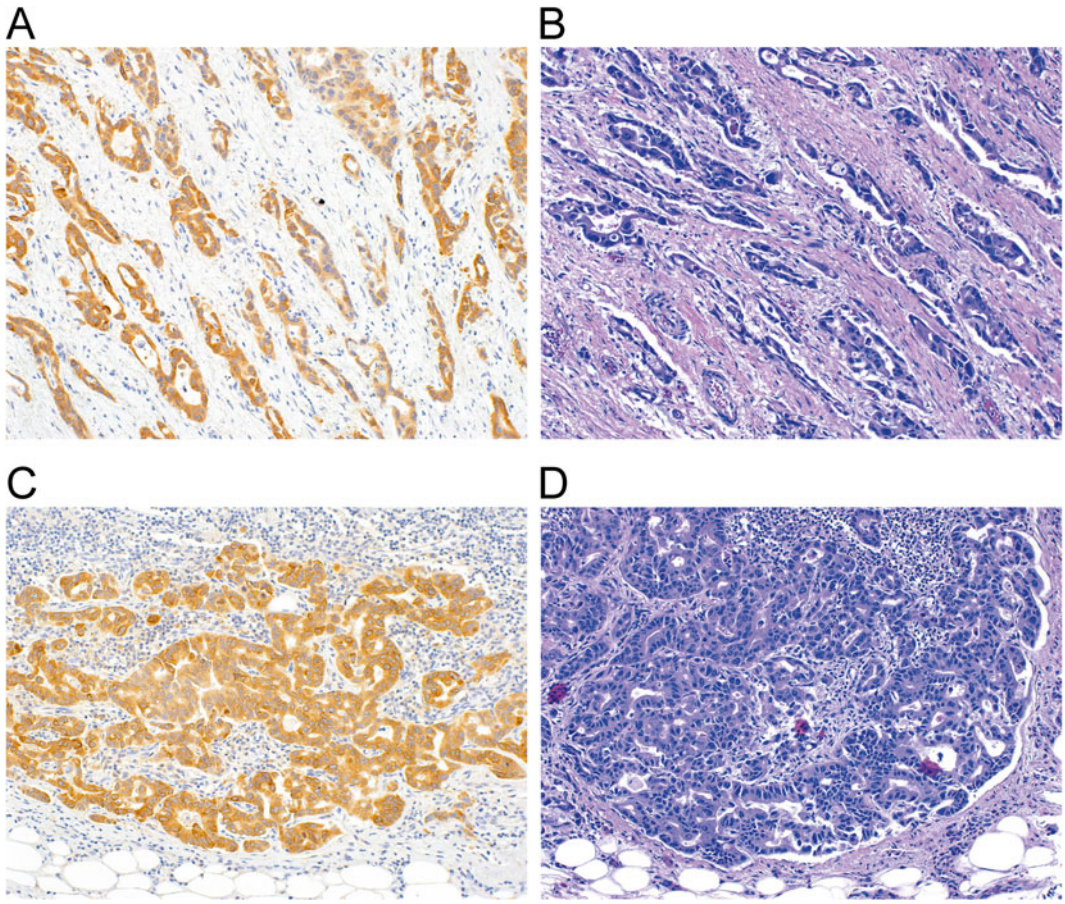

Fig. 4 IHC staining of LINE-1 ORF1p in pancreatic ductal adenocarcinoma. (**a**) and (**c**): Anti-ORF1p-stained sections with hematoxylin counterstain. (**b**) and (**d**): Hematoxylin and eosin (H&E) stain corresponding to (**a**) and (**c**), respectively

(c) Wash in 95 % ethanol for 2 min. Repeat twice for a total of three washes.

(d) Rinse under streaming tap water for 5 min.

2. *Antigen retrieval*:

Formalin and other aldehyde-based tissue fixations form extensive protein cross-links which require a subsequent antigen retrieval step for antibody penetration and antigen unmasking.

Our standard retrieval protocol uses a steamer as follows:

1. Immerse slides in citrate antigen retrieval buffer (pH 6).
2. Transfer the buffer and slides to a steamer and heat at 98 °C for 20 min.
3. Remove slides from steamer and allow them to cool to room temperature.
4. Place in TBST buffer.

An EDTA antigen retrieval buffer can be used in lieu of the citrate antigen retrieval solution.

In addition, the alternate hot plate approach below can be applied using either the citrate or the EDTA buffers. Thus, we provide four [4] different variations on conditions for antigen retrieval. Alternatively, several manufacturers make automated antigen retrieval systems using either heat or microwave energy; a review of these devices and their application is beyond the scope of this chapter. We suggest using the standard protocol above first and applying alternate protocols for optimization or based on the availability of heating equipment.

Alternate retrieval protocol: Hot plate approach

- Heat retrieval buffer to 55 °C.
- Immerse the rehydrated slides and increase the temperature to 95–98 °C.
- Incubate for 30 min.
- Let the slides cool to at least 45 °C in the antigen retrieval buffer. This step typically takes about 45 min.
- Rinse under streaming tap water for 5 min.

General note: The volume of each reagent used per slide from here on varies according to the surface area of the tissue. Reagent volumes can range between 100 μL and 1 mL per slide. It is recommended to add certain reagents (including blocking reagent, primary and secondary antibody solutions, chromogen and fluorophore mixes, and counterstains) dropwise to minimize their use.

3. *Permeabilization and blocking*:
   Two things are accomplished in this blocking protocol. First, activities of endogenous peroxidases are quenched. The protocol below uses a 3 % hydrogen peroxide solution (*see* **Note 13**). Secondly, blocking of nonspecific interactions is achieved using a 10 % non-immune serum blocking solution (Histostain kit) or BSA.
   1. Remove slides from TBST and wipe off any residual buffer on the slides using Kimwipes, gently drying the underside and edges. Take care to leave a small amount of buffer overlying the tissue, because drying the tissue during this step will interfere with antibody binding in subsequent steps.
   2. Incubate samples with 3 % hydrogen peroxide for 5 min at room temperature.
   3. Rinse slides with TBST (with shaking) for 5 min.
   4. Block:
      (a) Incubate samples with the blocking reagent (Reagent 1A, Histostain kit) at room temperature for 10 min.
      (b) Alternatively, overlay slides with 500–1000 μL of BSA-TBST blocking solution, and preincubate in a humid chamber for 30 min at room temperature (*see* **Note 14**).

4. *Primary Antibody Incubation*
    1. Remove slides from TBST and carefully wipe off any residual buffer on the slides.
    2. Dilute LINE-1 ORF1p antibody with BSA-TBST (TBST with 1 % BSA) to 1 μg/mL. The use of BSA reduces nonspecific binding of the primary antibody.
    3. Add enough diluted primary antibody to cover the tissue and incubate overnight at 4 °C in a humid chamber.
    4. Remove primary antibody solution and wash with TBST for 5 min. Repeat twice for a total of three washes.

Up to this point, the protocol has been identical for IF and IHC. We describe several approaches for subsequent staining:

- IF with fluorescent-conjugated secondary antibodies.
- IHC or IF with signal amplification using Histostain or EnVision reagents, followed by chromogenic or TSA-based IF detection.

Three starting points for these protocols follow:

A. Fluorescent secondary antibody incubation for IF
B. Secondary antibody incubation, chromogen incubation, and counterstain: Histostain Kit
C. Secondary antibody incubation, chromogen incubation, and counterstain: EnVision Kit

Departure points from each of the latter two methods to *amplified immunofluorescence using TSA* are noted. Finally, IHC detections conclude with *dehydration and coverslipping (for IHC only)*, and IF detections with *coverslipping (for IF only)* protocols.

A. Fluorescent secondary antibody incubation for IF
    1. Remove slides from TBST and carefully wipe off any residual buffer on the slides.
    2. Dilute fluorescent-conjugated secondary antibody or antibodies in BSA-TBST to 2 μg/mL (1:1000 for Alexa Fluor-conjugated goat antibodies but might change depending on the provider).
    3. Add enough diluted antibody to cover the tissue and incubate for 1 h at room temperature in a humid chamber.
    4. Remove the primary antibody solution and wash with TBST for 5 min. Repeat twice for a total of three washes.
    5. Proceed to *coverslipping (for IF only)*.
B. Secondary antibody incubation, chromogen incubation, and counterstain: Histostain Kit

1. Incubate slides with a biotinylated secondary antibody: Pipet Reagent 1B over tissue and incubate at room temperature for 10 min.
2. Remove the secondary antibody solution and wash with TBS (no tween) for 2 min. Repeat twice for a total of three washes.
3. Incubate slides with a streptavidin-peroxidase conjugate (Reagent 2) at room temperature for 10 min.
4. Remove the enzyme conjugate solution and wash with TBS (no tween) for 2 min. Repeat twice for a total of three washes.

If performing IF using the Histostain Kit, jump to the *amplified immunofluorescence using tsa (for IF only)* protocol below. Otherwise, for IHC, continue to **step 5** immediately below.

5. Incubate samples with DAB chromogen mix (one drop each of Reagents 3A, 3B, and 3C added to 1 mL water). The DAB chromogen mix should be protected from light and used immediately after mixing. The ideal development time for pancreatic tissue usually occurs at approximately 10 min but may vary across samples. Color development (brown) should be monitored using a light microscope.
6. Rinse with deionized water for 3 min with shaking. Repeat once for a total of two washes.
7. Incubate with the hematoxylin solution (Reagent 4). The ideal staining time for pancreatic tissue is approximately 7 min but may vary across samples.
8. Rinse with deionized water for 3 min with gentle shaking.
9. Proceed to *dehydration and coverslipping (for IHC only)*.

C. Secondary antibody incubation, chromogen incubation, and counterstain: EnVision™ Kit

1. Select the species-appropriate EnVision solution. Solutions are available with peroxidase-labeled polymers conjugated to anti-mouse or anti-rabbit antibodies.
2. Pipet the EnVision solution and incubate in a light-protected humid chamber for 1 h at room temperature (*see* **Note 15**).
3. Wash a total of three times with TBST.

If performing IF using the EnVisionKit, jump to the *amplified immunofluorescence using TSA (for IF only)* protocol below. Otherwise, for IHC, continue to **step 4** immediately below.

4. Prepare substrate-chromogen solution which consists of one drop (20 μL) of DAB+ chromogen added to 1 mL DAB+ substrate buffer. Use this solution immediately after mixing.
5. Wipe excess liquid off of the slides and pipette DAB mixture onto each slide.
6. Rinse with deionized H_2O for 3 min with shaking. Repeat once for a total of two washes.
7. Wipe excess liquid off of the slides and pipet on a small amount of hematoxylin, enough to cover the tissue surface, and incubate at room temperature for 1 min.
8. Rinse slides in deionized H_2O for 3 min with gentle shaking.
9. Proceed to *dehydration and coverslipping (for IHC only)*.

5. *Dehydration and coverslipping (for IHC only)*.
   1. Sequetially dehydrate samples by immersing them in the following series of increasing ethanol concentrations and ending with xylene:
      (a) Water, 5 min.
      (b) 70 % ethanol, 2 min.
      (c) 95 % Ethanol, 2 min. Repeat once for a total of two 95 % EtOH soaks.
      (d) 100 % Ethanol, 2 min. Repeat twice for a total of three 100 % EtOH soaks.
      (e) Xylene for 3 min, 5 min, and 5 min (fume hood).
   2. Remove slides from xylene and wipe excess solution of the underside and sides of the slides; as before, make sure not to allow the tissue to dry entirely.
   3. Add a few drops of HistoMount, CytoSeal 60, or other mounting medium and cover slip immediately (*see* **Note 16**).
6. *Amplified immunofluorescence using TSA (for IF only)*.
   1. Remove TBST, and wipe slides carefully with Kimwipes, making sure not to dry out the tissue.
   2. Dilute fluorophore-tyramide 1:50 (Cy-5) or 1:100 (fluorescein) in amplification diluent and pipet enough reagent to cover tissue.
   3. Incubate in the dark at room temperature for 10 min.
   4. Rinse slides in 1× TBST for 5 min with shaking. Repeat once.
   5. Proceed to *coverslipping (for IF only)*.
7. *Coverslipping (for IF only)*.
   1. Aliquot 50 to 100 μL of ProLong Gold with 4′,6-diamidino-2-phenylindole (DAPI) solution onto slides.

2. Incubate in the dark at room temperature for 5 min (*see* **Note 17**).
3. Place a cover slip cleaned with a Kimwipe or an air can duster carefully onto slides (*see* **Note 18**).

4 Notes

1. PFA is toxic. Weigh and dissolve in a fume hood and wear appropriate protection.
2. Antibodies can be obtained from several suppliers. Different Anti-Flag antibodies can be obtained and Mouse Anti-Flag M2 Sigma F3165 is not affinity purified but works well in our hands for this application.
3. The drying process is important for surface stickiness.
4. *It is critical not to let the cover slips dry out.* They should be without buffer for the shortest time possible. When working with many samples, aspirate one 6-well plate at a time, wash that plate, and then move to the next samples.
5. Far-red is not visible to the eye and requires charge-coupled device (CCD) camera-based detection.
6. We have had good success using Alexa-fluor-labeled highly cross-adsorbed goat antibodies from Life Technologies, with combinations of anti-mouse-rabbit-chicken, anti-mouse-rabbit-rat, and anti-mouse-rabbit-sheep.
7. *Helpful trick*: If more than one combination of primary antibody is being used (or if any volume needed to pipet is less than 0.5 μL), it is helpful to first make 2× working stocks of each antibody (or 3× stocks for three-channel immunostaining).
8. If using plastic dishes, pinch firmly to free the cover slip.
9. It is helpful if the forceps have a very slight upward curve, to prevent stabbing into the Parafilm. If the tip of the instrument is getting stuck in the Parafilm, try using the other tip (flip them over), or gently bend the end inward.
10. Commercial mounting media such as VECTASHIELD (Vector labs) and ProLong (Life Technologies) are popular and more permanent, but some require solvents and longer drying times, and all are more expensive.
11. Alternatively, either of the Histostain or EnVision™ HRP-based amplification systems can be used with the Tyramide Signal Amplification™ (TSA™) System (PerkinElmer, Inc., Waltham, MA) for increased sensitivity. A variety of fluorescent substrates are available for TSA™; we mostly use TSA™-plus fluorescein and TSA™-plus Cyanine-5 (Cy-5). Multiplexed immunofluorescent detection using TSA and EnVision is described in [20].

12. Ink from many laboratory markers will wash off in ethanol.
13. An alternative is to use Peroxo-Block™ (Invitrogen Cat. No. 00-2015).
14. We make the humid chamber using a small sealable plastic container, lined on the bottom with damp paper towels and containing an elevated stage to hold the slides.
15. The binding time is a step that can be optimized for each primary antibody.
16. Use gloves during this step, because any fingerprint(s) on the cover slip may interfere with subsequent microscopic analysis. Allow slides to dry in hazardous chemical hood, label with permanent marker, and store slides at room temperature.
17. Keep slides protected from light from the beginning of this step and onwards. Other mounting media (as described in the cultured cell IF protocol above) can be used here as well.
18. Use gloves during this step, because any fingerprint(s) on the cover slip may interfere with subsequent microscopy. Label slides with permanent marker. Store slides shielded from ambient light at room temperature or 4 °C.

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