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## Method Article

# Identification of invadopodia by TKS5 staining in human cancer lines and patient tumor samples

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## A B S T R A C T

Invadopodia, cancer cell protrusive structures with associated proteolytic activity, provide cancer cells with the ability to remodel the extracellular matrix. Invadopodia facilitate invasive migration and their formation correlates with cancer cell invasiveness and metastatic potential. The unambiguous identification of invadopodia is an important step to undergo studies on invadopodia regulatory inputs, functional outputs, as well as the prevalence and significance of invadopodia for cancer cells and human tumors. The adaptor protein TKS5 is a known invadopodia regulatory protein, which is necessary for invadopodia formation and activity. TKS5 is highly enriched at invadopodia and, unlike other commonly used invadopodia markers, it does not accumulate significantly in other types of cellular protrusions. However, the use of TKS5 as a marker of invadopodia has not been generalized, in part due to the availability of suitable antibodies against the human protein. We have evaluated two commercial antibodies raised against human TKS5. Here, we detail protocols for the detection of invadopodia-associated TKS5 in human cells in culture and in paraffin-embedded archived tumor surgical specimens using commercial antibodies. These methods should facilitate the identification and study of human invadopodia.

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- TKS5 staining identifies invadopodia in human cancer cell lines and archived surgical tumor specimens.

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#### Method details

##### *Method 1: TKS5 immunofluorescence staining in cultured cancer cells*

Here, we describe a protocol for the analysis of TKS5 localization at invadopodia in the human pancreatic adenocarcinoma line BxPC-3 [1]. This cell line requires the presence of an extracellular matrix component (such as gelatin) to fully induce invadopodia formation in vitro. Other cell lines may form invadopodia when grown directly on glass, or require additional matrices or growth factor supplements to readily form invadopodia. The adequate conditions to induce invadopodia formation, therefore, are cell-type dependent and need to be identified experimentally. Detection of invadopodia using TKS5 staining can be combined with the detection of F-actin using a phalloidin fluorescent conjugate, or with the detection of focal proteolysis using a gelatin fluorescent conjugate as a substrate to grow cells. Here, we described the staining of TKS5 and F-actin in BxPC-3 cells growing on unlabeled gelatin B. We have previously described a detailed protocol for the preparation of fluorescent gelatin coverslips and analysis of invadopodia activity [2,3], which can be used in combination with TKS5 immunofluorescence [4,5].

##### *Equipment*

- Tissue culture incubator and Biosafety Cabinet.
- Water-bath or thermomixer with 65 °C heating capacity
- Vacuum set up
- Rocking platform
- Fluorescent microscope equipped with appropriate filters and 63× objective.

##### *Materials*

- BxPC-3 cells, cat. no. CRL-1687 (ATCC, Manassas, VA)
- Cover Glasses, round, 18 mm diameter, cat. no. 12-548A (ThermoFisher Scientific, Waltham, MA).
- Sterile 12-well multi-well plates, cat. no. 712001 (Bioland Scientific, Paramount, CA)

- Forceps, fine tip, 5 in., cat. no. 3120019 (ThermoFisher Scientific, Waltham, MA).
- Glass slides, cat. no. 12550003 (ThermoFisher Scientific, Waltham, MA).
- Parafilm, cat. no.1337410 (ThermoFisher Scientific, Waltham, MA).

### Reagents

- Cell culture medium (RPMI-1640), cat. no. MT10040CV (Corning, Corning, NY).
- Fetal bovine serum (FBS), cat. no. MT35015CV (Corning, Corning, NY).
- % Ethanol (diluted from 190 proof Ethanol), cat. no. 04-355-226 (ThermoFisher Scientific, Waltham, MA).
- 0.05% Trypsin-EDTA, cat. no. 25-052-CI (Corning, Corning, NY).
- Penicillin/Streptomycin solution, 100× cat. no. 30-002-CI (Corning, Corning, NY).
- Gelatin B, 2% solution, cat. no. G1393 (MilliporeSigma, St. Louis, MO).
- Tissue culture water, cat. no. MT25055CV (Corning, Corning, NY).
- Poly-Lysine solution 0.1 mg/ml, cat. no. 5049 (Advanced Biomatrix, Carlsbad, CA).
- Glutaraldehyde 25%, cat. no. 00376-500 (Polysciences, Warrington, PA).
- Sodium Borohydride, cat. no. S678-25 (ThermoFisher Scientific, Waltham, MA).
- Triton X-100, cat. no. 64-846-650ML (MilliporeSigma, St. Louis, MO).
- Phosphate buffer saline (PBS), cat. no. 21040CV (Corning, Corning, NY).
- Bovine Serum Albumin, fraction V, cat. no. BP1600-100 (ThermoFisher Scientific, Waltham, MA).
- Paraformaldehyde 16%, cat. no. 15710 (EMS, Hatfield, PA).
- Anti-human TK5 antibody (clone 13H6.3), cat. no. MABT336 (MilliporeSigma, St. Louis, MO).
- Alexa 488-conjugated goat anti-mouse IgG, highly cross-adsorbed, cat. no. A11029 (ThermoFisher Scientific, Waltham, MA).
- Alexa 568-conjugated phalloidin, cat. no. A12380 (ThermoFisher Scientific, Waltham, MA).
- Mounting Medium with DAPI, cat. no. H-1200 (Vector Laboratories, Burlingame, CA).

### Recipes

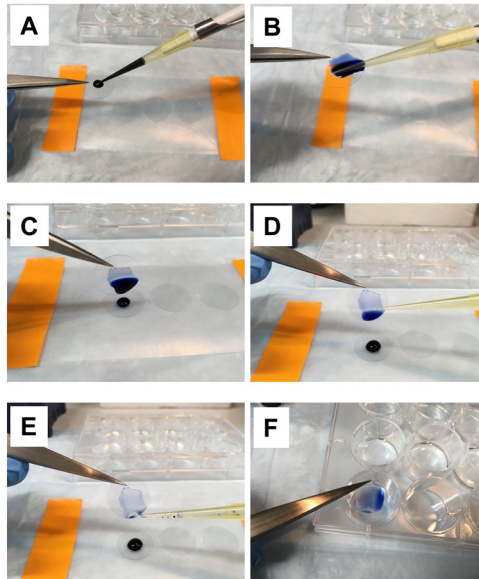
- Washing Buffer (PBS-T): PBS containing 0.01% Triton X-100
- Blocking buffer: washing buffer containing 3% BSA
- Antibody incubation buffer: washing buffer containing 0.3% BSA

### Procedure

#### Step 1: preparation of coverslips (1–2 days)

##### *Poly-D-lysine coating*

1. Working inside a biosafety cabinet, and using sterile forceps, sterilize glass coverslips by submerging them in 70% Ethanol for 30 s. Place them into the wells of a sterile 12-well plate. Wash 3 times with sterile tissue culture water.
2. Add 0.5 ml of sterile poly-D-lysine solution (0.1 mg/ml, undiluted) to each well. Secure the lid to the plate with parafilm or tape to maintain sterility, and rock for 5–10 min on a rocking platform at room temperature.
3. Return the plate to the biosafety cabinet and remove the poly-D-lysine solution.
4. Let poly-D-lysine dry overnight at room temperature, or for 2 h at 37 °C. *NOTE: Poly-NULL-lysine solution can be reused 5 or 6 times upon storage in sterile conditions at 4 °C. Poly-NULL-lysine-coated coverslips can be stored at 4 °C for up to 3–4 weeks.*



**Fig. 1.** Steps for coating coverslips with gelatin. Illustration of the procedure of coating coverslips with gelatin B. Please note that instead of gelatin solution, which is naturally translucent-to-white and viscous, the illustration uses a blue-dye solution for a clearer visualization of the procedure. (A) Coverslip is held horizontally while solution is pipetted on top. (B) Using the side of the pipette tip, the solution is spread over the coverslip. (C) The coverslip is then tilted to let the solution drop onto the next coverslip. (D, E) Holding the coverslip vertically, aspirate excess solution from the bottom edge of the coverslip. (F) Place the coverslip into a multiwell plate to proceed with the next steps.

### Gelatin coating

1. Prepare glutaraldehyde fixing solution (0.5% final concentration in PBS). Keep in ice. *NOTE: Use glutaraldehyde inside a fume hood, and dispose according to Institutional Environmental Hazard Safety regulation.*
2. Prepare Sodium Borohydride solution (5 mg/ml in PBS). *NOTE: We recommend to weigh the amount of powder that will be used (calculate for 2 ml well), and keep in powder form in a conical tube. Right before is needed, dilute in PBS (see step 6). Dispose according to your Institutional Environmental Hazards Safety regulation. Containers holding sodium borohydride should not be tightly sealed until all gas pressure has been released.*
3. Using poly-D-lysine-coated coverslips prepared as indicated above, proceed to coating the coverslips with gelatin B following the next sequential steps (see also Fig. 1):
  - a Warm Gelatin B solution stock (2%) to 65 °C and keep it at 65 °C throughout the procedure.
  - b Place a rectangular piece of parafilm affixed to the bench top with tape.
  - c Place 4 coverslips on top of the parafilm piece in a row.
  - d Open a 12-well plate (no sterility is needed yet).
  - e Open vacuum system (i.e. flask connected with a hose holding a glass pasteur pipette for liquid aspiration), avoiding a strong vacuum suction.
  - f Hold one coverslip horizontally with forceps using your non-dominant hand, and pipette 100  $\mu$ l of gelatin on top of it (Fig. 1A). Using the side of the plastic pipette tip, distribute the gelatin over the glass (Fig. 1B).
  - g Tilt the coverslip and let the excess gelatin pour onto the next coverslip placed on top of the bench (Fig. 1C). Still holding the coverslip vertical with forceps and your non-dominant hand, carefully aspirate the excess gelatin from the bottom edge of the coverslip (Fig. 1D, E). Try to avoid the gelatin sticking to the other side of the coverglass.
  - h Place coverslip into a well of the 12-well plate.

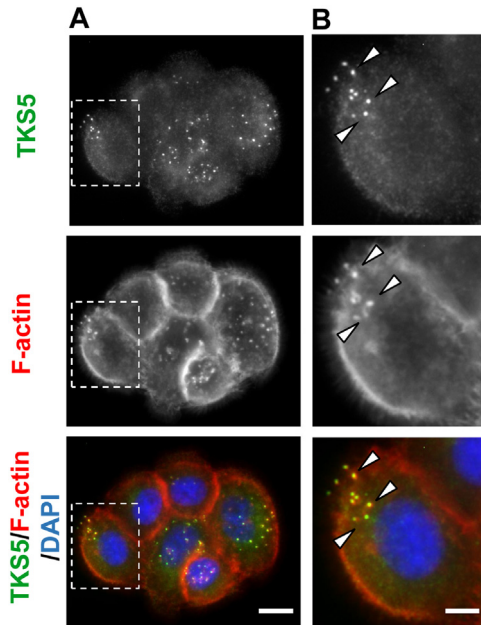
- i Repeat with each of the remaining coverslips. For the last one, instead of tilting the gelatin solution onto another coverslip, collect it with the pipette and return it to the stock. Keep the stock warm at 65 °C while preparing the next set of 4 coverslips, and repeat the procedure using ~100 µl of gelatin per each set of 4 coverslips. *NOTE: Preparing batches of 4 coverslips ensures that gelatin is warm enough to perform a uniform coating.*
- j Let them dry at room temperature for about 1–2 h. *NOTE: We recommend to inspect the plate to make sure that coverslips are not sticking to the bottom of the plastic well. If so, carefully lift the coverslip using forceps and move it to another plate before gelatin solidifies.*
4. Add 1 ml glutaraldehyde solution per well and incubate for 15 min on ice.
5. At room temperature, wash each well with PBS 3 times for 5 min. *NOTE: No rocking is necessary for efficient washing in this protocol, but a rocking platform may be used if desired.*
6. Before removing the last wash, dissolve sodium borohydride powder in PBS. Aspirate PBS from wells and add 2 ml of sodium borohydride solution per well. Incubate for 3 min. *NOTE: Make sure coverslips remain submerged, because sodium borohydride bubbling can make them float.*
7. Wash each well 3 times for 5 min with PBS.
8. Transfer plate to the biosafety cabinet. Use sterile forceps to carefully transfer all coverslips to a new sterile 12-well plate.
9. Wash 3 times for 5 min with sterile PBS. Coverslips may be used immediately, or stored at 4 °C in PBS or RPMI containing 2× Pen/Strep for up to 3 weeks.

#### Step 2: cell plating and incubation (1 day)

1. Working inside a biosafety cabinet, prepare the number of gelatin-coated coverslips necessary for the experiment by placing them in a 12-well sterile plate. Wash them 3 times for 5 min with 1 ml of sterile PBS to remove pen/strep..
2. Aspirate PBS and add 1 ml of growing medium (RPMI containing 10% FBS) per well. Equilibrate inside a tissue culture incubator for about 30–60 min. While medium is equilibrating, trypsinize and count BxPC-3 cells. Plate 1 ml of cell suspension on top of the gelatin-coated coverslips at a plating density of around 80,000 cells per well. Incubate for 18–24 h at 37 °C in a humidified atmosphere containing 5% CO<sub>2</sub>. *NOTE: The incubation time necessary to observe invadopodia-mediated gelatin degradation needs to be empirically determined for each cell type.*

#### Step 3: cell staining (2 days)

1. Transfer the plate with cells from the tissue culture incubator to the benchtop, and carefully aspirate medium.
2. Wash cells once with PBS.
3. Remove PBS and add to each well 0.5 ml of 4% paraformaldehyde solution freshly prepared in PBS. Incubate for 15 min at room temperature. *NOTE: Use paraformaldehyde inside a fume hood, and dispose according to your Institutional Environmental Hazards Safety Regulations.*
4. Wash cells three times for 5 min with PBS and incubate in Blocking Buffer for 1 h at room temperature.
5. Wash cells once with Washing Buffer, and add 0.5 ml/ well of Antibody Solution Buffer containing 2 µg/ml of TKS5 antibody. Incubate overnight at 4 °C. *NOTE: Optimal TKS5 antibody concentration needs to be experimentally determined for each cell type*
6. Wash cells three times for 10 min with Washing Buffer, and incubate with 0.5 ml/ well of Antibody Dilution Buffer containing anti-mouse IgG-Alexa 488 (4 µg/ml) and Phalloidin-Alexa 568 (0.4 U/ml).. Incubate for 1 h at room temperature protected from light. *NOTE: Samples need to be protected from light from now on.*
7. Wash cells 3 times for 10 min with Washing Buffer and 2 times for 10 min with PBS. Mount coverslips on glass slides using DAPI-containing Mounting Medium.
8. Visualize Images under a fluorescent microscopy. Images in [Fig. 2](#) were obtained using a 63× objective in a Zeiss AxioImager A1 equipped with a PRIOR Lumen 200, AxioCam 503 mono camera



**Fig. 2.** TKS5 immunofluorescence staining of pancreatic adenocarcinoma BxPC-3 cells. BxPC-3 cells were incubated for 24 h on gelatin-coated coverslips, and stained with anti-TKS5 monoclonal antibody (13H6.3) to identify invadopodia (arrowheads). Samples were co-stained with phalloidin conjugated with Alexa 568 to reveal F-actin. Slides were mounted in medium containing DAPI to visualize nuclei. (A) BxPC-3 cells stained with the indicated markers. (B) Magnification of the area enclosed by a rectangle in A. Bar, 20  $\mu\text{m}$  in (A) and 8  $\mu\text{m}$  in (B).

and ZEN2 software. *NOTES: Coverslips may be sealed to the slides with nail polish. Immunostained samples may be stored at 4 °C in the dark for several weeks, but it is recommended to acquire images as soon as possible after completing the staining procedure.*

## Anticipated results

Approximately 30–50% of BxPC-3 cells growing for 18–24 h on gelatin-covered coverslips should present invadopodia, which are identified as ventral TKS5-positive puncta colocalizing with discrete F-actin puncta (Fig. 2 and [4]). When fluorescent gelatin is used as a substrate to grow cells, TKS5-positive puncta often co-localize with active degradation areas identified as black areas within the fluorescent background [4]. These puncta represent mature invadopodia. TKS5 is also a marker of immature invadopodia and therefore a fraction of TKS5-positive invadopodia is expected to lack colocalization with gelatin degradation at a given time [4]. When using a cancer cell line for the first time to identify invadopodia formation, it is advisable to stain TKS5 along with F-actin as described here, and also to stain TKS5 in cells growing on fluorescent gelatin to verify extracellular matrix degrading invadopodia activity.

### Method 2: TKS5 immunofluorescence staining in paraffin embedded tumor sections

This method has been valuable to identify for the first time invadopodia inside formalin-fixed paraffin-embedded archived tumor samples [4,5]. Tumors from various organs were obtained from patients treated at Harbor-UCLA Medical Center, under protocol 31,363 approved by the Institutional Review Board at Los Angeles Biomedical Research Institute.

### Equipment

- Microwave oven
- Fluorescent microscope equipped with appropriate filters and 100× objective using Nikon morphometric measurement system of fluorescent intensity.

### Materials

- Paraffin sections of human tumor surgical specimens (5 μm thickness).
- Coplin jars (plastic, autoclavable).
- Glass coverslips, cat. no. 2980245 (Corning, Corning, NY).

### Reagents

- Xylene, cat. no. X3S-4 (ThermoFisher Scientific, Waltham, MA)
- 100% Ethanol, cat. no. A962-4 (ThermoFisher Scientific, Waltham, MA)
- Double Distilled water
- Phosphate Buffer saline (PBS), pH 7.4
- Antigen Retrieval Solution (AR Citra, pH6), cat. no. HK086 (BioGenex, San Ramon, CA)
- Normal donkey serum, cat. no. 017-000-121 488 (Jackson ImmunoResearch Laboratories, West Grove, PA)
- Human TKS5 rabbit polyclonal antibody, cat. no. LS-C383498 (LifeSpan BioSciences, Seattle, WA)
- Donkey anti-rabbit IgG Alexa Fluor 488 (Jackson ImmunoResearch Laboratories, West Grove, PA)
- 4',6-Diamidine-2'-phenylindole dihydrochloride (DAPI), D1306 (ThermoFisher Scientific, Waltham, MA)
- *p*-Phenylenediamine cat. no. P6001 (MilliporeSigma, St. Louis, MO).
- Glycerol, cat. no. 9012 (MilliporeSigma, St. Louis, MO).

### Recipes

- Mounting medium: *p*-Phenylenediamine 0.1% in PBS and glycerol (1:9).

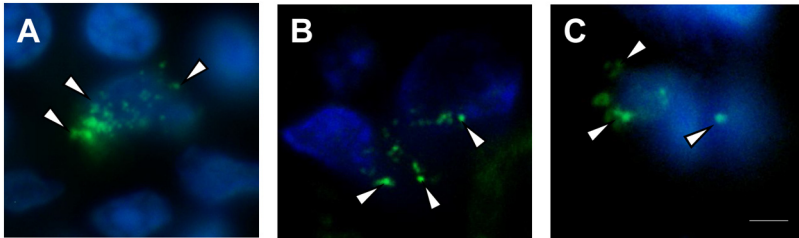
### Procedure

#### Step 1: slide deparaffinization and rehydration

- Place paraffin slides sequentially in the following solutions:
- Xylene, 2 washes, 5 min each
- 100% Ethanol, 1 wash, 5 min
- 95% Ethanol, 1 wash, 5 min
- 70% Ethanol, 1 wash, 5 min
- PBS, 2 washes, 5 min each

#### Step 2: antigen retrieval and blocking

1. Transfer slides to a microwavable coplin jar containing 1 × retrieval solution prepared in water. Use enough solution to cover the slides through the procedure of heat-induced antigen retrieval (around 50 ml depending on the container used). Bring samples to a rapid boiling at high power (500–1000 W), and then let simmer at 200 W for 15 min. *NOTE: Microwaving conditions may need to be adjusted depending on microwave wattage, temperature and volume of the antigen retrieval solution.*
2. Let cool down for 30 min, and wash 3 times with PBS for 5 min each.



**Fig. 3. Representative images of TKS5-positive invadopodia-like puncta in human tumor samples.** Formalin fixed paraffin-embedded invasive pancreatic ampullary adenocarcinomas from three different patients (A–C) were processed for immunofluorescence staining with an antibody against human TKS5 (LSBio). Images were obtained at 100× magnification. Arrowheads indicate invadopodia-like puncta. Bar, 0.5 μm.

3. Block slides in 5% normal donkey serum prepared in PBS for 1 h at room temperature.
4. Wash 3 times with PBS for 5 min each.

#### Step 3: Antibody incubation and detection

1. Incubate slides with anti-TKS5 (20 μg/ml in PBS) overnight at 4 °C. *NOTE: Optimal TKS5 antibody concentration needs to be experimentally determined for each sample.*
2. Wash 2 times with PBS for 5 min each.
3. Incubate slides with anti-rabbit IgG-Alexa Fluor 488 (20 μg/ml in PBS) for 1 h at room temperature. Protect samples from light for the rest of the experimental procedure and during storage.
4. Wash two times with PBS for 5 min.
5. Incubate with DAPI (300 nM in PBS) for 10 min at room temperature.
6. Wash 3 times with PBS for 5 min each.
7. Mount with cover glasses using mounting medium. .
8. Visualize using a fluorescence microscope. For images in Fig. 3, we used a 100x oil-immersion objective in a Nikon eclipse E400 microscope with Excelitas X-Cite 120 fluorescence illuminator, a Nikon Y-IDP Double port, and a Nikon DS-Fi2 camera head. Nikon NIS elements D imaging software was used for image acquisition. *NOTES: It is recommended to include an isotype IgG staining as a negative control on each staining procedure.*

#### Anticipated results

TKS5 staining pattern is expected to be membranous and cytoplasmic, but the intensity of the staining will be variable depending on TKS5 protein abundance in each particular tumor sample. In order to visualize invadopodia-like structures, tumor areas need to be carefully analyzed at 100× magnification. TKS5-positive puncta corresponding to invadopodia-like structures, often appear close to the nuclei and display a polarized distribution (Fig. 3 and [4]). We have successfully used TKS5 immunofluorescence of human surgical tumor specimens in combination with Cortactin staining, which is a commonly-used marker of invadopodia [4,5].

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