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3D Gel Invasion Assay of Gastric Cancer Cells with Fibroblasts

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[Abstract] Cancer tissue is composed of cancer cells and a large number of stromal cells including fibroblasts. In order to understand the relationship between fibroblasts and cancer cells during invasion of the stroma, 3D gel invasion assay is useful. Most tumors are associated with a biologically active type of fibroblasts known as cancer-associated fibroblasts (CAFs), which promote the invasion of cancer cells. Here, we describe the method of imaging the invasion by fluorescently labeled CAFs and gastric cancer cells in gels containing extracellular matrix. For two-color fluorescence labeling of living cells, long-chain dialkylcarbocyanines, DiO and DiI were used. This method is also applicable for studying invasion by other stromal cells and cancer cells, and for evaluation of drugs targeting cancer stromal cells.

Materials and Reagents

1. Collagen-coated dish (Sanyo, IWAKI, catalog number: 4010-010)
2. Transparent PET membrane 24 well 3.0 μm pore size (Corning, Falcon®, catalog number: 353096)
3. 24 well plate for use with cell culture inserts (Corning, Falcon®, catalog number: 353504)
4. Razor blades (Esbjerg, Feather, catalog number: FA-10)
5. Microslide glass (Matsunami Glass Ind, catalog number: TF0215M)
6. Micro cover glass, 24 x 24 mm (Thickness NO.1: 0.12-0.17 mm) (Matsunami Glass Ind)
7. Gastric cancer cells (44As3) (Yanagihara *et al*., 2005)
   Note: It's established from gastric cancer patient.
8. Fibroblasts (CAF) (Fuyuhiro *et al*., 2011)
   Note: It's isolated from surgical materials of gastric cancer patients.
9. Type I-collagen (Nitta Gelatin Inc., Cellmatrix Type I-P)
10. Matrigel matrix (Corning, catalog number: 356234)
11. 3, 3'-dioctadecycloxacarbo-cyanine perchlorate (DiO) (Thermo Fisher Scientific, Molecular Probes™, catalog number: D-275)
12. 1, 1'-dioctadecyl-3, 3, 3', 3'-tetramethyllindo-carbocyanine perchlorate (DiI) (Thermo Fisher Scientific, Molecular Probes™, catalog number: D-282)

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13. Trypsin-EDTA solution (Sigma-Aldrich, catalog number: T3924)
14. Penicillin-Streptomycin (Sigma-Aldrich, catalog number: P4333)
15. Dulbecco's modified Eagle's medium (Sigma-Aldrich, catalog number: D6046)
16. RPMI-1640 medium (Sigma-Aldrich, catalog number: R8758)
17. 10x Dulbecco's modified Eagle's medium (Sigma-Aldrich, catalog number: D2429)
18. 10x RPMI-1640 medium (Sigma-Aldrich, catalog number: R1145-500ML)
19. Fetal bovine serum (heat inactivated) (Sigma life science, catalog number: 172012-500ML, batch: S13C490)
20. Phosphate buffered saline (PBS) (Sigma-Aldrich, catalog number: P4417)
21. 4% Paraformaldehyde phosphate buffer (Wako Pure Chemical Industries, catalog number: 163-20145)
22. Polyvinyl alcohol mounting medium (Sigma-Aldrich, Fluka, catalog number: 10981)
23. Instant glue (Krazy Glue, catalog number: KG585)
24. $NaHCO_3$ (Sigma-Aldrich, catalog number: S6014-500G)
25. NaOH (Sigma-Aldrich, catalog number: S5881-500G)
26. HEPES (Sigma-Aldrich, catalog number: H7006-25G)
27. Gel (0.2 mg/ml type I-collagen and 2.5 mg/ml matrigel matrix) (see Recipes)
28. Reconstitution buffer (see Recipes

Equipment

1. Tweezers (Electron Microscopy Sciences, Dumont, model: No.5)
2. 37 °C, 5% CO_2 incubator (LabX, Sanyo, model: MCO-19AIC)
3. Dissecting microscope (OLYMPUS CORPORATION, model: SZ61)
4. Light source (OLYMPUS CORPORATION, KL1600LED)
5. Vibratome (DOSAKA EM, model: LinearSlicer PRO7)
6. Confocal laser scanning microscope (ZEISS, model: LSM 780)

Procedure

1. 44As3 cells and gastric CAFs were cultured in RPMI-1640 medium or DMEM, respectively supplemented with 10% FBS and Penicillin (100 unit/ml)-Streptomycin (0.1 mg/ml) at 37 °C in a humidified atmosphere containing 5% CO_2. CAFs were maintained in collagen-coated dishes.
2. 44As3 cells and CAFs were labeled with DiO or DiI, respectively as follows. Stock solutions of DiO and DiI were prepared in dimethylformamide at 2.5 mg/ml. DiO or DiI was added in the medium at final concentration of 6 μg/ml, and cells were incubated for 1 h in a CO_2 incubator. Cells were then washed by replacing with fresh medium containing 10% FBS and backed into a CO_2 incubator for 20 min, repeat this step two cycles.

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3. During labeling the cells, prepare the gel. Serum-free DMEM/RPMI-1640 (1:1) medium containing 0.2 mg/ml type I-collagen and 2.5 mg/ml matrigel matrix (refer to the below recipe) was laid onto the upper chamber of transwells (150 µl/ well), and solidify in a CO_2 incubator at 37 °C for 30 min.
4. 44As3 cells and CAFs were detached by trypsin-EDTA (trypsin 0.5 g/L, incubate 3 min) and mixed (1.5 x 10^4 cells each) in 200 µl of medium composed by 1:1 mixture of DMEM and RPMI-1640 supplemented with 0.2% FBS, and placed on gels. The lower compartment of the transwell was filled with 700 µl of the same medium with 10% FBS. Cells were incubated for 5-9 days with replacing the upper and lower medium every other day (Figure 1).

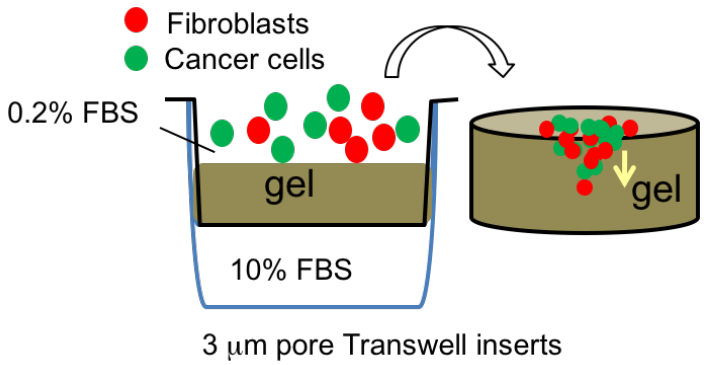

Figure 1. Cell-culture on the gel in transwell inserts. (Left). Mixture of cancer cells and fibroblasts, labeled with distinguishable fluorescence dyes were put on the gel, which was prepared in 3.0 µm pored transwell inserts. (Right). The cells invade into the gel according to serum gradient.

5. Take the gels out from transwell inserts by cutting the insert membrane along the whole circumference (Video 1). The gels were fixed in 4% paraformaldehyde for 1 h at room temperature, or overnight at 4 °C. If necessary, take photos of the whole gel as activated fibroblasts contract the gel (Figure 2A).
6. The fixed gel was once rinsed with PBS. Periphery of the gel was excised by a razor blade under dissecting microscope (Figure 2B). The edge of cell area, which is usually visible by labeled fluorescence is excised. The gel was attached to the stage of vibratome slicer by instant glue (Figure 3A, B), and cut into 200 µm thick slices in PBS (Figure 3C-D, Video 2).

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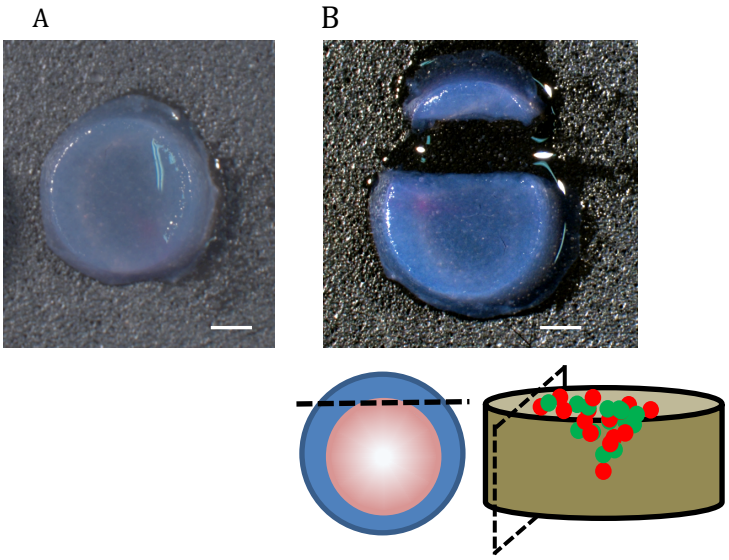

Figure 2. Appearance of the gel before setting on the vibratome. A. Appearance of the whole gel after taking out from the insert and fixation. B. After excision of peripheral part of the gel. The position of excision was shown at the bottom.

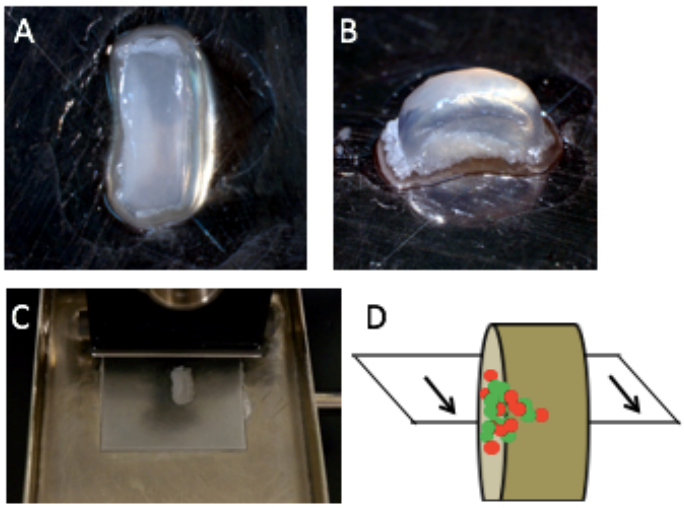

Figure 3. Slice of the gel by a vibratome. A, B. Attach the gel face a section down to the metal stage of a vibratome. A. Superior view, B. Lateral view of the gel. C. The stage is filled with PBS. The gel is sectioned by a razor blade in PBS. D. Direction of the sectioning was shown.

7. The slices were picked up from PBS, put on a glass slide, and fix the shape of the slices by straightening them with tweezers under dissecting microscope. Wipe excess PBS by a paper and mounted in polyvinyl alcohol mounting medium (Figure 4).

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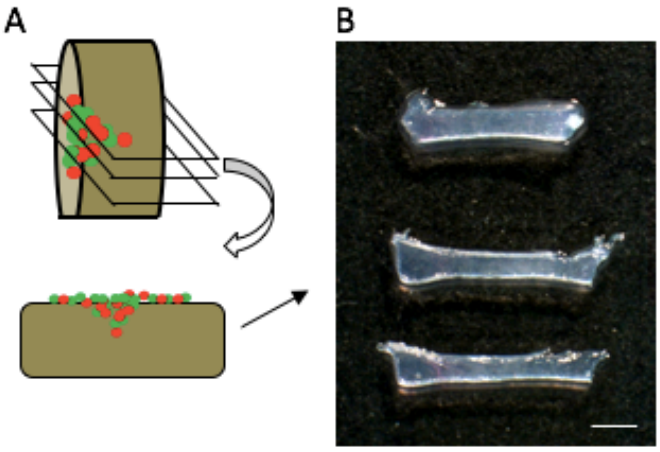

Figure 4. Appearance of sliced gels. A. Illustrations of sliced gels. B. Sliced gels were arranged on a slide glass. Bar = 1 mm

8. After solidified the mounting medium, the slices were visualized using a confocal microscope. The 3D-rendered images are obtained from the z-stack images (Figure 5).

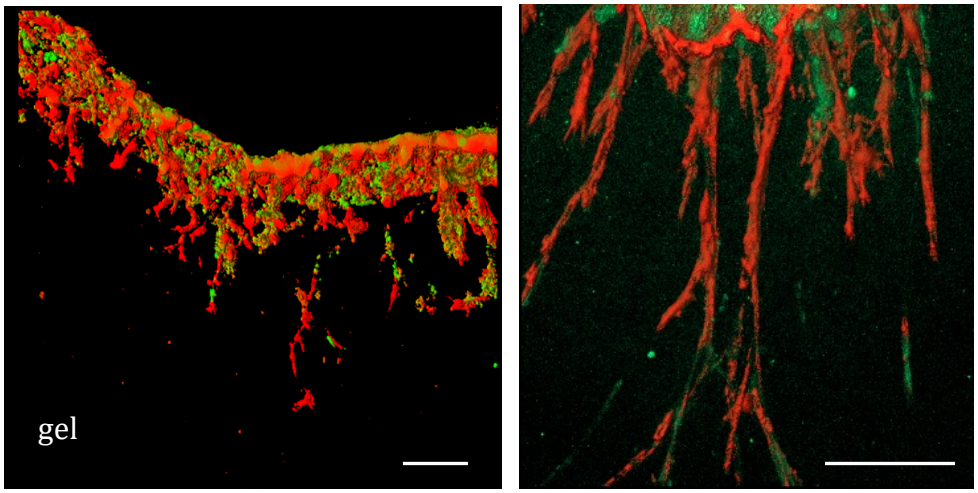

Figure 5. Representative confocal images of a gel section. Green: Cancer cells, Red: CAFs. Mixture of cancer (44As3) cells and CAFs protruded and invaded into the gel. Gels were fixed at day 7. Bar = 100 μm

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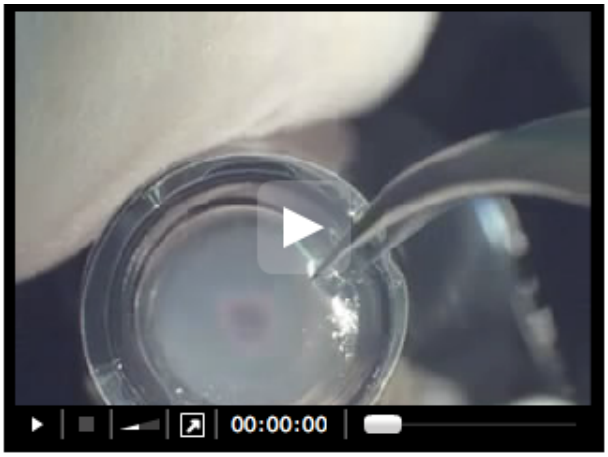
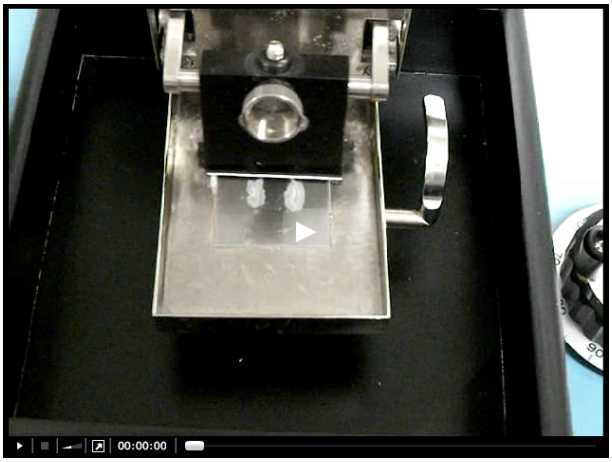
Representative data

Video 1. Take the gels out from transwell inserts by cutting the insert membrane



Video 2. Gel sectioning by a vibratome. In this video, two gels are attached to the stage.



Notes

The optimum length of culture period should be determined empirically. Usually between 5-9 days. If solidified glue was attached to the sliced gel, it should be removed in PBS using tweezers under dissecting microscope before mounting.

Recipes

1. Gel (0.2 mg/ml type I-collagen and 2.5 mg/ml matrigel matrix)
   Mix type-I collagen gel and matrigel matrix (3:1). For example, if you make total 800 µl mixed gel, at first prepare 600 µl of type-I collagen by mixing 10x conc. culture medium (60 µl), reconstitution buffer (60 µl) and type-I collagen (480 µl) in a tube on ice. Add 200 µl of matrigel matrix and mix thoroughly.

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2. Reconstitution buffer
   2.2 g $NaHCO_3$ in 100 ml of 0.05 N NaOH and 200 mM HEPES

Acknowledgments

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