TRIPLE (Insulin, Glucagon and EGFP) Immunofluorescence Staining Protocol in Pancreas

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[Abstract] This protocol aims to introduce methods for immunostaining two endogenous proteins insulin and glucagon and one exogenous transgene driven EGFP in mouse pancreatic islet. The immunostaining results of insulin and glucagon indirectly tell functionality of pancreatic beta cells and alpha cells respectively. Furthermore, the protocol provides immunostaining steps for the third protein which can be applicable to any other endogenous proteins with a specific antibody generated in mouse.

Materials and Reagents

1. Mouse pancreas
2. Ethanol (100%, 95%, 85%, 70%, 50%)
3. 0.85% NaCl
4. ddH_2O
5. 99.5% Xylene (Sigma-Aldrich, catalog number: 534056)
6. 10% Formalin solution (neutral buffered) (Sigma-Aldrich, catalog number: HT501128)
7. Bovine Serum Albumin (BSA) (Sigma-Aldrich, catalog number: A7906)
8. Tween 20 (Sigma-Aldrich, catalog number: P9416)
9. Triton-X 100 (Sigma-Aldrich, catalog number: T8787)
10. Guinea pig anti-insulin antibody (EMD Millipore, catalog number: 4011-01F)
11. Rabbit anti-glucagon antibody (EMD Millipore, catalog number: 4030-01F)
12. Mouse anti-EGFP antibody (Clonetech, catalog number: 632381)
13. Texas Red conjugated donkey anti-guinea pig antibody (Jackson ImmunoResearch Laboratories, catalog number: 106-075-003)
14. Alexa 350 conjugated goat anti-rabbit antibody (Life Technologies, Invitrogen™, catalog number: A-21068)
15. FITC conjugated goat anti-mouse antibody (Jackson ImmunoResearch Laboratories, catalog number: 715-095-166)
16. ProLong Gold Antifade Reagent (Life Technologies, Invitrogen™, catalog number: P36935)

17. 10x PBS (Gibco®, catalog number: 70011-044)
18. Blocking solution (see Recipes)

Equipment

1. Glass jar
2. Humidified chamber
3. Confocal microscope
4. Tissue cassette

Procedure

A. Formalin Fixation
   1. Fix overnight pancreas (~5mm x ~5mm) removed from a mouse in a 15 ml conical tube by 10 ml 10% formalin solution during rotating it at 4 °C.
   2. On next day, transfer the fixed tissue to a tissue cassette and store it in 70% Ethanol solution till making paraffin block (for at least 1 day) at room temperature.
   3. Make paraffin blocks of fixed tissues.
   4. Make slide section of paraffin blocks by Microtome.

B. Immunostaining of slide sections
   1. Deparaffinization step
      a. Keep sections for 5 min in 99.5% Xylene solution 2 times.
      b. Keep sections for 10 min in 99.5% Xylene solution 2 times.
      c. Keep sections for 3 min in 100% ethanol with new glass jar.
      d. Keep sections for 3 min in 95% ethanol.
      e. Keep sections for 3 min in 85% ethanol.
      f. Keep sections for 3 min in 70% ethanol.
      g. Keep sections for 5 min in 50% ethanol.
      h. Keep sections for 5 min in 0.85% NaCl with new glass jar.
      i. Keep sections for 5 min in 1x PBS.
   2. Permeabilization step
      a. Incubate sections for 30 min in 0.1% Triton-X 100 in PBS with new glass jar at room temperature (RT).
      b. Incubate sections for 5 min in 1x PBS with new glass jar 3 times.
   3. Blocking, Primary and Secondary Antibody (Ab) binding steps
      a. Aspirate around the tissue until the slide, not the tissue, is dry. Carefully, trace around the tissue with a grease choke.
      b. Block for 30 min at RT with 2% BSA in PBS with 0.05% Tween 20 in a coplin jar.
      c. Aspirate blocking buffer and incubate sections in a humidified camber with

glucagon primary Ab (1/100 dilution), in 2% BSA in PBS with 0.05% Tween 20 for overnight (o/n) at 4 °C. Use enough antibody solution (~150 µl) to completely submerge the section.

d. Aspirate primary antibody and wash sections for 5 min in 2% BSA in PBS with 0.05% Tween 20 2 times in a coplin jar.

e. Aspirate washing buffer and incubate sections in a humidified chamber with corresponding secondary Alexa350 conjugated goat anti-rabbit Ab (1/500 dilution) for 1.15 h at RT in dark. Dilute secondary Ab in 2% BSA in PBS with 0.05% Tween 20. Use enough antibody solution (~150 µl) to completely submerge the section.

f. Aspirate secondary antibody and wash sections for 5 min in 2% BSA in PBS with 0.05% Tween 20 2 times in a coplin jar. Aspirate around the tissue until the slide, not the tissue, is dry. And incubate sections in a humidified chamber with Insulin primary Ab (1/500 dilution), in 2% BSA in PBS with 0.05% Tween 20 for 1.5 our at RT in dark. Use enough antibody solution (~150 µl) to completely submerge the section.

g. Aspirate primary antibody and wash sections for 5 min in 2% BSA in PBS with 0.05% Tween 20 2 times at RT in dark in a coplin jar.

h. Aspirate around the tissue until the slide, not the tissue, is dry. And Incubate sections in a humidified chamber with corresponding secondary Texas Red conjugated donkey anti-guinea pig Ab for 1 h at RT in dark. Dilute secondary Ab (1/1,000 dilution) in 2% BSA in PBS with 0.05% Tween 20. Use enough antibody solution (~150 µl) to completely submerge the section.

i. Aspirate secondary antibody and wash sections for 5 min in 2% BSA in PBS with 0.05% Tween 20 2 times at RT in dark in a coplin jar. Aspirate around the tissue until the slide, not the tissue, is dry. And incubate sections in a humidified camber with EGFP primary Ab (1/500 dilution), for 1 h at 4 °C in dark. Diluted primary Ab in 2% BSA in PBS with 0.05% Tween 20. Use enough antibody solution (~150 µl) to completely submerge the section.

j. Aspirate primary antibody and wash sections for 5 min in 2% BSA in PBS with 0.05% Tween 20 2 times at RT in dark in a coplin jar.

k. Aspirate washing buffer and Incubate sections in a humidified chamber with corresponding secondary FITC conjugated goat anti-mouse Ab (1/500 dilution) for 1 h at RT in dark. Dilute secondary Ab in 2% BSA in PBS with 0.05% Tween 20. Use enough antibody solution (~150 µl) to completely submerge the section.

l. Aspirate secondary antibody and wash sections for 5 min in 2% BSA in PBS with 0.05% Tween 20 2 times at RT in dark in a coplin jar

C. Mounting Step

1. Aspirate around the tissue until the slide, not the tissue, is dry, Add ~50 µl of ProLong® Gold Antifade Reagent containing DAPI to each section and place a cover

slip over section.

Note: Be careful to avoid damaging the tissue by sliding the cover slip too much and do not introduce bubbles. Aspirate excess reagent.

2. Dry sections at room temperature for several hours or overnight protected from light.
3. Once the slides are completely dry, seal the edges of the cover slip with clear nail polish. Slides can be stored at -20 °C, protected from light for several weeks.

Recipes

1. Blocking solution (2% BSA in PBS pH 7.4 with 0.05% Tween20)
   10 g BSA
   50 ml 10x PBS
   0.25 ml Tween20
   Add ddH_2O to 500 ml

Representative Data

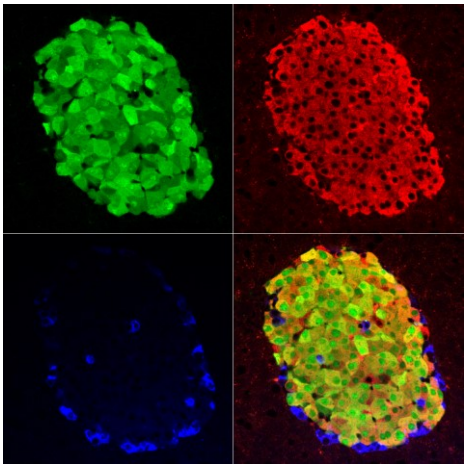

Figure 1. Triple immunostaining for EGFR, insulin, and glucagon in a pancreatic tissue section. Pancreatic tissue sections were obtained from mice 3 weeks after Tam administration to induce EGFP from a transgene. Pancreatic tissue sections were triple immunostained for EGFP (Green), insulin (Red), and glucagon (Blue), and representative single-channel fluorescence images are shown individually and merged (lower-right image of each group). Please cite Reference 1 (Figure 1B).

Acknowledgments

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HL52173, and HL057346 (R.J.K.). R.J.K. was an Investigator of the Howard Hughes Medical Institute. This protocol was adapted from Back *et al.* (2009), and a first short version of the adapted protocol was published in Han *et al.* (2013).

References

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