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An end-to-end workflow for GD2 immunofluorescence staining in unfixed human neuroblastoma tissue samples

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Sara Peggion[1], Clara Volz[1], Magdalena Trochimiuk[1], Isabelle Ariane Bley[2,3], Júlia Ramos[4], Konrad Reinshagen[1], Laia Pagerols Raluy[1]

[1]Department of Pediatric Surgery, University Medical Centre Hamburg-Eppendorf, Martinistrasse 52, 20246 Hamburg, Germany;
[2]Division of Pediatric Stem Cell Transplantation and Immunology, Clinic of Pediatric Hematology and Oncology, University Medical Center Hamburg-Eppendorf, 20246 Hamburg, Germany;
[3]Research Institute Children's Cancer Center Hamburg, 20251 Hamburg, Germany;
[4]Institut Bonanova, Circumval.lació 8, 08003 Barcelona, Spain

S Sara Peggion
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Abstract

This protocol allows the staining of the membrane marker GD2 **directly** in unfixed tumor tissue samples prior to 3D and/or 2D fluorescence microscopy.
By using this workflow it will be possible to avoid extracellular epitope loss (and consequent false negative results), which is experienced in the immunostaining of FFPE tissue slices due to initial tissue treatment.

Guidelines

The protocol was developed according to the Ordinance on Safety and Health Protection at Workplaces Involving Biological Agents (Biological Agents Ordinance - BioStoffV) of 15 July 2013 (Federal Law Gazette [BGBl.] Part I p. 2514), last amended by Article 1 of the Ordinance of 21 July 2021 (Federal Law Gazette I p. 3115).
Full text can be found at the following link:
https://www.gesetze-im-internet.de/englisch_biostoffv/englisch_biostoffv.html

Materials

Plastic/metallic ware

- Pipette tips for battery-operated pipetting aid (5-10ml)
- Lab spoons (potentially also stainless steel, autoclavable)
- Magnetic stir bar(s)
- Cryogenic tubes with screw cap (2ml are sufficient)
- Petri plate (90×14,2mm)
- Disposable scalpel
- tweezers
- Variable pipette tips (1-1000µL)
- 96 well assay plate with black lateral walls and clear bottom with lid (*e.g. Corning Incorporated - Ref. No. 3603)*
- Variable safe-lock tubes (1-50mL)
- Variable brown safe-lock tubes (1-50mL)
- Aluminium foil *(e.g. LabSOLUTE, Cat.No. 7 696 857)*

Glassware

- Beaker (e.g. Pyrex® Griffin beakers, low form, with printed trace code)
- Variable graduated laboratory bottle with cap, non-sterile, autoclavable

Equipment

- Battery-operated pipetting aid
- Precision balance
- Magnetic stirrer
- Research lab refrigerator (for 4°C storage)
- Laboratory freezer (for -20°C storage)
- Laboratory deep freezer (for -80°C storage)
- Laboratory (fume) hood
- Laboratory bench
- Cryogenic liquid nitrogen container
- Variable microliter pipettes (1-1000µL)
- Mini-centrifuge
- Vortex-mixer
- (Digital) orbital shaker
- (Portable digital) laboratory timer
- Paraffin station

Reagents and buffers

- Fetal Bovin Serum *(e.g. Gibco™ Fetal Bovine Serum, Ref. No. A5256801)*
- Bovine Serum Albumin *(e.g. Sigma-Aldrich, Cat. No.* A9418)
- DPBS *(Dulbecco's Phosphate Buffered Salin - Gibco™, Ref. No. 14190-094)*
- Freezing medium for tissue specimens *(Recovery™ Cell Culture Freezing Medium, Gibco - Cat. No. 12648-10)*

- Purified Mouse anti-Human Disialoganglioside GD2 – Clone 14.G2a *(BD Pharmingen™, Cat. No. 554272)* - 0,5mg/mL
- Purified Mouse IgG2a, κ Isotype Control *(BD Pharmingen™, Cat. No. 556651)* - 0,5mg/mL
- Donkey Anti-Mouse IgG H&L conjugated with the dye Alexa Fluor® 647 *(abcam, Cat. No. ab150107)* - 2mg/mL
- DAPI *(e.g. Roth, Art. Nr. 6335.1)*
- Ultrapure distilled water *(e.g. Sigma Aldrich, Cat.No. 38796-1L)*
- 4% formaldehyde with carbonate buffer/neutral pH *(e.g. Engelbrecht, Cat.No. 10192)*
- Paraffin

Safety warnings

Use the laboratory hood according to manufacturer instructions in order to keep proper laboratory professionals' safety while working with biological material.

Ethics statement

This protocol was developed in agreement with the declaration of Helsinki on the use of human material for research. Ethical approval was obtained by the local ethics committee of Hamburg (No. 2020-10041-BO-ff). Make sure that written informed consent of all patients or their custodians is obtained in case you are going to use human tissue samples obtained during diagnostic biopsy, etc.
Experiments involving animals must be have obtained approval from an Institutional Animal Care and Use Committee or equivalent.

Before start

Ensure a lab coat, lab goggles (if necessary) and plastic gloves are worn throughout the whole protocol performance.

Preliminary steps

1h

1 **2% FCS/1% BSA - washing buffer preparation:**

Dissolve e.g. 2 mL FCS *(Gibco™ Fetal Bovine Serum, Ref. No. A5256801)* with 1 g BSA *(Bovine Serum Albumin - Sigma-Aldrich, Cat. No.* A9418) in 98 mL DPBS *(Dulbecco's Phosphate Buffered Salin - Gibco™, Ref. No. 14190-094).*

Mixing by using a magnetic stirrer is suggested; BSA has to be completely dissolved before usage.

Note

FCS has not to be sterile, but must be stored at 4°C. Otherwise it is possible to thaw FCS, which has been long-term stored at -20°C or -80°C.
BSA has not to be sterile either, but must be stored at 4°C.
Surplus washing buffer can be stored in a closed container at 4°C for up to 2 weeks.

The above mentioned amounts are merely a given example, the exact needed amounts should be calculated depending on tissue sample quantity and used assay plate; by using a 96 well assay plate the needed volume of washing buffer and further reagents can be minimized (150 µL **per well).**

2 **Tissue sample preparation:**

Safety information

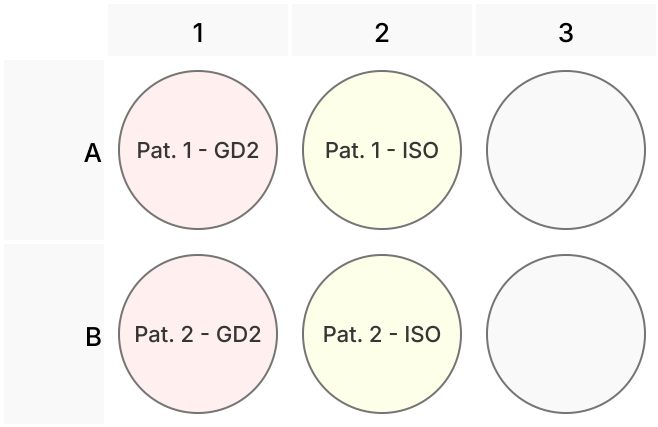
Throughout the staining protocol (possibly untested) tissue should be manipulated under laboratory hood conditions.

Note

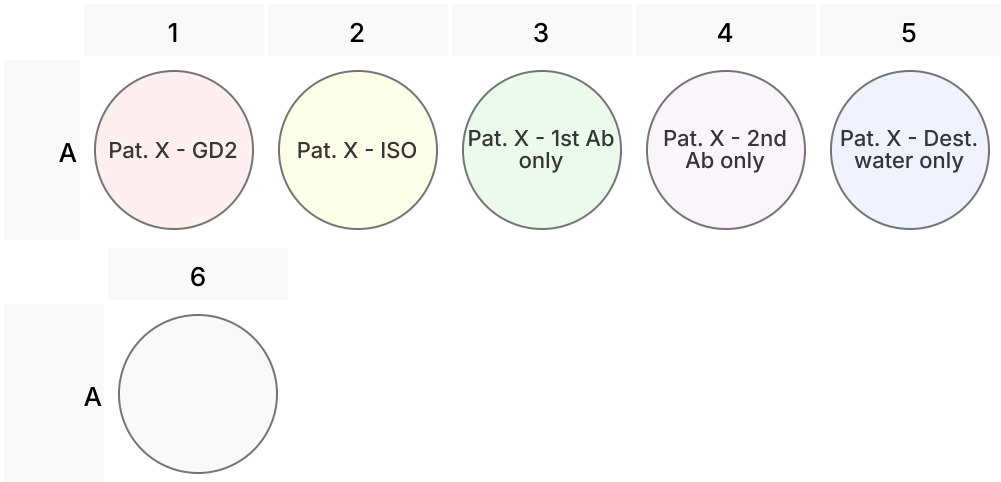
Either fresh tissue (preserved in NaCl 0,9% solution upon collection) or thawed frozen tissue can be stained with this protocol. Tissue cryopreservation (in liquid nitrogen) should have been performed using freezing medium (*e.g. Recovery™ Cell Culture Freezing Medium, Gibco - Cat. No. 12648-10*) for optimal tissue characteristic maintenance.

Specimens with max. 3 mm thickness shall be prepared after tissue washing with DPBS (500-1000 µL - depending on sample size) and placed separately in a 96 well assay plate with black lateral walls and clear bottom with lid (*e.g. Corning Incorporated - Ref. No. 3603*).
Dark lateral walls assure optimal protection against light exposure when incubation with a fluorochrome conjugated antibody is performed.

Following scheme proposes a possible staining design according to antibody type and patient's sample:



Following scheme proposes a completion of the previous staining design with adjunctive negative controls:



Signal detection after tissue samples' incubation with only either primary or secondary antibody as well as only with ultrapure distilled water (e.g. *Sigma Aldrich, Cat.No. 38796-1L*) undermines staining results's reliability.

3 **Antibody dilution:**
Primary antibodies - stored at 4°C, according to manufacturer's instructions

- Purified Mouse anti-Human Disialoganglioside GD2 – Clone 14.G2a (BD Pharmingen™, Cat. No. 554272) - [M] 0,5 mg/mL
- Purified Mouse IgG2a, κ Isotype Control (BD Pharmingen™, Cat. No. 556651) - [M] 0,5 mg/mL

Secondary antibody - stored at -20°C, according to manufacturer's instructions

- Donkey Anti-Mouse IgG H&L conjugated with the dye Alexa Fluor® 647 (abcam, Cat. No. ab150107) - [M] 2 mg/mL

3.1 ***Primary antibody dilution:***

Note

After titration tests the optimum antibody concentration with strong positive signal and minimum background or nonspecific reactions was shown to be [M] 0,0025 mg/mL , which means a 1/200 dilution starting from the above mentioned antibody concentration.

Prepare the appropriate volume of DPBS **(consider the example below)** in a safe-lock tube (e.g. *Eppendorf Safe-Lock Tubes*).
Spin down the undiluted primary antibody for up to 2 seconds before pipetting it into DPBS. Pipette the right amount of the selected primary antibody into the appropriate volume of DPBS. Vortex the obtained diluted antibody solution for up to 2 seconds.
Repeat these steps in order to analogously dilute the isotype control antibody.
Store diluted antibody solutions at 4°C until usage.

Practical dilution example:
4 wells x 150µL/well = 600µL (tot. vol.) → 3µL antibody + 597µL DPBS

3.2 ***Secondary antibody dilution:***

Note

After titration tests the optimum antibody concentration with strong positive signal and minimum background or nonspecific reactions was shown to be [M] 0,01 mg/mL , which means a 1/200 dilution starting from the above mentioned antibody concentration.

Let the antibody thaw at room temperature.
Prepare the appropriate volume of DPBS in a **brown** safe-lock tube (e.g. *Eppendorf Safe-Lock Tubes - brown*), in order to avoid photobleaching.
Spin down the thawed undiluted secondary antibody for up to 2 seconds before pipetting it into DPBS. Pipette the right amount of the selected secondary antibody

into the appropriate volume of DPBS. Vortex the obtained diluted antibody solution for up to 2 seconds.
Store diluted antibody solutions at 4°C until usage.

Practical dilution example:
4 wells x 150µL/well = 600µL (tot. vol.) → 3µL antibody + 597µL DPBS

4 **DAPI solution (4',6-diamidino-2-phenylindole) preparation**

Dilute DAPI (*e.g. Roth, Art. Nr. 6335.1*) in ultrapure distilled water (*e.g. Sigma-Aldrich, Cat. No. 102539811*) in order to reach a concentration of [M] 1 µg/mL by pipetting up and down in a **brown** safe-lock tube (e.g. *Eppendorf Safe-Lock Tubes)*, in order to avoid photobleaching. Subsequently**,** vortex the tube containing DAPI diluted solution for up to 2 seconds.

Note

Undiluted DAPI must be stored at -20°C and thawed at room temperature upon dilution. Surplus DAPI diluted solution can be stored in a closed container at 4°C for up to 6 months (avoiding exposure to light sources).

Tissue staining

5m

5

Note

Following steps require a a 200µL-pipette and appropriate tips.

1st washing step

Add 150 µL of the previously mixed washing buffer (go to step #1) to each well containing a tissue specimen. Cover the 96 well assay plate. Complete this first washing step by placing the assay plate on an orbital shaker at 20-30 rpm, Room temperature for 5 minutes.

6 **2nd washing step**

8m

Discard the washing buffer. Repeat step 5 (go to step #12).

Safety information

Discard washing buffer appropriately.

7 **Protein blocking**

20m

Discard the washing buffer. Add 150 µL of the previously mixed washing buffer (go to step #4) to each well containing the tissue specimens and incubate them on an orbital shaker at 20-30 rpm, Room temperature for 20 minutes, after covering the 96 well assay plate.

Note

Before any antibody application it is necessary to perform adequate protein blocking on tissue samples to prevent antibodies from binding to non-target epitopes.

Citation

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doi: 10.1007/978-1-4939-8935-5_26 LINK

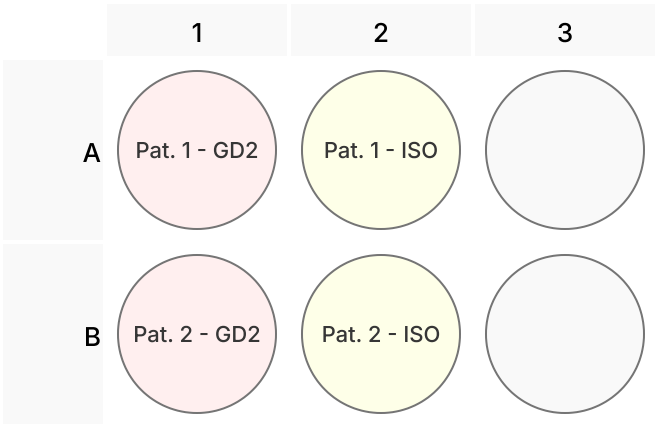
8 **Incubation with the primary antibody**

Discard the washing buffer.

Incubate each target tissue specimen with 150 µL of the previously diluted primary antibody (go to step #4) on an orbital shaker at 20-30 rpm for 4 hours at room temperature.

Note

For each human sample undergoing the staining protocol two specimens should be obtained. One specimen is incubated with diluted Purified Mouse anti-Human Disialoganglioside GD2 – Clone 14.G2a (BD Pharmingen™, Cat. No. 554272); the second one serves as an appropriate negative control by being incubated with diluted Purified Mouse IgG2a, κ Isotype Control (BD Pharmingen™, Cat. No. 556651), as displayed in the following *Well Plate Map*.



9 **3rd washing step**

Discard the primary antibody solution. Add 150 µL of the previously mixed washing buffer (go to step #1) to each well containing a tissue specimen. Cover the 96 well assay plate. Incubate on an orbital shaker (20-30 rpm, Room temperature) for 5 minutes.

10 **4th washing step**

Discard the washing buffer. Repeat step 9 (go to step #12).

11 **Incubation with the secondary antibody**

Discard the washing buffer.

Incubate each target tissue specimen with 150 µL of the previously diluted secondary antibody (go to step #4) on an orbital shaker at 20-30 rpm for 90 minutes at room temperature.

Note

From now on it is mandatory to protect the plate from light sources by wrapping it with aluminium foil (e.g. *LabSOLUTE, Cat.No. 7 696 857*).

12 **5th washing step**

Discard the secondary antibody solution. Add 150 µL of the previously mixed washing buffer to each well containing a tissue specimen. Cover the 96 well assay plate. Incubate on an orbital shaker (20-30 rpm, Room temperature) for 10 minutes.
Do not forget to keep the assay plate protected from light sources by wrapping it with aluminium foil.

13 **6th - 7th washing step**

Discard the washing buffer. Repeat step 12 twice (go to step #12).

Note

These washing steps (00:10:00 repeated for three times) reduce unspecific secondary antibody binding at a minimum and therefore downscale unintended background signal.

14 **Nuclei staining with DAPI (4′,6-diamidino-2-phenylindole)**

Discard the washing buffer. Add 150 µL of the previously diluted DAPI solution (go to step #4) to each well containing a tissue specimen. Cover the assay plate. Complete incubation by placing the assay plate on an orbital shaker at 20-30 rpm, Room temperature for 30 minutes.

15 **9th washing step**

Discard DAPI solution and wash tissue specimens by adding 150 µL of DPBS to each well. Cover the assay plate and incubate the samples on an orbital shaker at 20-30 rpm, Room temperature for 5 minutes.

Safety information

Discard DAPI solution appropriately.

16 **10th washing step**

Discard DPBS and wash tissue specimens by adding 150 µL of ultrapure distilled water (e.g. *Sigma Aldrich, Cat.No. 38796-1L*) to each well. Cover the assay plate and incubate the samples on an orbital shaker at 20-30 rpm, Room temperature for 5 minutes.

17 **Tissue fixation**

Discard distilled water. Add 150 µL of 4% formaldehyde with carbonate buffer/neutral pH (e.g. *Engelbrecht, Cat.No. 10192*) to each well containing a tissue specimen. Cover the assay plate. Formaldehyde fixation (at RT) over 1 hour is sufficient for thin tissue specimens (max. 3 mm) . In case of thicker tissue specimens, fixation may be prolonged by storing the samples at 4°C overnight.

Note

Formaldehyde fixed tissue can directly undergo confocal fluorescence microscopy in order to obtain 3D images and screen rapidly for GD2 expression pattern. Since most laboratories do not have direct access to a confocal microscope, tissue can be further paraffin embedded. FFPE tissue slices can be analyzed by mean of standard 2D fluorescence microscopy.
Detailed information about these further procedures can be found in: Peggion, S.; Volz, C.; Trochimiuk, M.; Bley, I.A.; Ramos, J.; Reinshagen, K.; Pagerols Raluy, L. - A Backwards Approach to GD2 Immunofluorescence in Human Neuroblastoma Tissue Samples: From Staining to Slicing. Cells **2025**, 14, 1462. https://doi.org/10.3390/cells14181462

Citations

Step 7

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doi: 10.1007/978-1-4939-8935-5_26

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