

Chapter 2

Detecting Gene Expression in Lymphoid Microenvironments by Laser Microdissection and Quantitative RT-PCR

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Abstract

Quantitative reverse transcription-polymerase chain reaction (qRT-PCR) is a valuable tool for measuring gene expression in cells and tissues. Unique challenges are encountered when studies are performed on cells microdissected from small specific areas of frozen animal or human tissue. This chapter describes the analysis of gene expression of chemokines and cytokines that are important for the differentiation and migration of germinal center (GC) derived plasmablasts/plasma cells and memory B cells by using laser capture microdissection (LCM) and qRT-PCR to examine tissue sections.

Key words Quantitative RT-PCR, Laser capture microdissection, Germinal center, B cells

1 Introduction

Exposure to vaccines or pathogens results in the activation of B lymphocytes. This ultimately leads to the production of high affinity antibodies that are protective against invading antigens. After antigen exposure, activated B lymphocytes migrate through different microenvironments in lymphoid tissues, where they mature into high affinity antibody forming cells—the plasma cell [1]. Maturation to high affinity happens in a specialized microenvironment: the germinal center (GC). This maturation process represents a very rapid Darwinian evolution at a cellular level: involving B cell proliferation, hypermutation of the genes that encode antibody, and the antigen-dependent selection of B cells with genetic information coding for the highest affinity antibodies [2].

How activated B lymphocytes travel through the different microenvironments, which signals (including molecular and cellular signals) regulate the GC reaction, and which signals regulate the production and migration of plasma cells and memory cells from GC remains unanswered. Techniques such as flow cytometry,

quantitative gene expression analysis, and others are able to provide precise information to answer these questions. However, some types of cells in tissue are still not so easy to isolate by traditional techniques, for example, the stroma that surrounds differentiating lymphocytes in lymphoid tissues. Some chemokines and cytokines are produced at a specific time and location. The location of signals involved in the differentiation of GC B cell, plasma cells, and memory B cells can be identified by defining the local environment by histology and isolating small areas of tissues.

Laser microdissection is a powerful tool for the isolation of targeted cell populations (or even single cells) from stained sections of both formalin-fixed, paraffin-embedded, and frozen tissues. The material is suitable for a wide range of downstream assays, particularly gene expression analysis as to mRNA [3, 4]. This chapter describes the use of laser capture microdissection from frozen sections, and the protocol of qRT-PCR analysis using small amounts of mRNA from laser microdissected lymphoid tissue microenvironments.

2 Materials

2.1 Immunization (See Note 1)

1. Chicken gamma globulin (CGG; e.g., Jackson ImmunoResearch).
2. Alum $\text{AlK}(\text{SO}_4)_2$ (e.g., Sigma).
3. 10 M NaOH.
4. Heat inactivated *Bordetella pertussis* (b.p.) bacteria (e.g., BD Lee Laboratories).
5. NP-O-succinimide ester (NP-OSu) (e.g., LGC BioSearch Technologies)
6. Dimethylformamide (DMF).
7. 0.1 M sterile sodium bicarbonate (NaHCO_3).
8. 10,000 MW dialysis cassettes (e.g., Slide-A-Lyzer™, Thermo Fisher)

2.2 Immunohistology of Adjacent Tissues

1. Tissue-Tek OCT compound (e.g., Sakura).
2. Cryospray (e.g., Bright).
3. Four-spot polytetrafluoroethylene (PTFE)-coated glass slides (e.g., Hendley-Essex).
4. Acetone.
5. Glass wash trough.
6. Humid chamber to incubate slides.
7. Wash buffer: mix 1 Vol 0.2 M Tris (Trizma base), 1.4 Vol 0.1 N HCl, 1.6 Vol 0.855 NaCl. Wash buffer should have pH 7.6.

8. Normal mouse serum (e.g., Sigma).
9. 3,3'-Diaminobenzidine hydrochloride (DAB) tablets (e.g., Sigma), H₂O₂. 1 tablet dissolved in 15 ml Tris-HCl (pH 7.6).
10. Alkaline phosphatase (AP) substrate Fast Blue: dissolve 8 mg levamisole hydrochloride (e.g., Sigma) in 10 ml Tris-HCl (pH 9.2). Mix with 340 µl of DMF containing 4 mg naphthol AS-MX phosphate. Finally, add 10 mg Fast Blue BB salt and shake until dissolved. Filter and use immediately.
11. Glycerol gelatin as mounting medium.
12. 22-mm × 65-µm glass coverslips.
13. Cryostat (e.g., Bright).
14. Goat anti-mouse IRF4 (e.g., Santa Cruz Biotechnology).
15. Rat anti-mouse IgD (e.g., BD Bioscience).
16. Biotinylated donkey anti-sheep goat (e.g., Binding Site).
17. Peroxidase (PX) labeled rabbit anti-rat (e.g., DakoCytomation).

2.3 Cresyl Violet Staining of Microdissected Tissues

1. PALM membrane slides NF (polyethylene naphthalate (PEN) membrane coated slides) from Zeiss.
2. PALM Liquid Cover Glass N from Zeiss.
3. Cresyl violet acetate (e.g., Sigma).
4. Ethanol (RNA free) for molecular biology.
5. Injection water (e.g., B. Braun).
6. RNase-free water.
7. Slide scanner (e.g., AxioScan Z1, Zeiss).

2.4 Microdissection

1. Eight-well strips of 200 µl PCR tubes (e.g., Alpha laboratories).
2. Eight-well cap stripes (e.g., ABgene™, Thermo Fisher).
3. Lysis buffer: RLT buffer from Rneasy® Micro Kit from Qiagen.
4. Low Biding barrier pipette tips (20 µl) (e.g., Multiguard).
5. Dry ice.
6. PALM Microbeam HT microscope from Zeiss.

2.5 RT-PCR

1. RNase Free DNase.
2. RNeasy Micro Kit from Qiagen.
3. Shredder spin column from Qiagen.
4. Microcentrifuge allowing max speed 15871 × g.
5. Random hexanucleotide primers.
6. 100 mM dNTP.

7. RNase Inhibitor (e.g., RNasin Plus, Promega).
8. 200 U/ μ l Moloney murine leukemia virus (M-MLV) reverse transcriptase (e.g., Invitrogen) containing $5 \times$ first strand buffer, 0.1 M DTT.
9. 96-well PCR machine.
10. Real-Time PCR master Mix (e.g., TaqMan Universal PCR master Mix, Applied Biosystems).
11. Clear adhesive foil (e.g., Applied Biosystems).
12. Real-time PCR probes for genes of interest and housekeeping genes, such as β 2-microglobulin (e.g., Applied Biosystems).
13. Real-Time PCR System with 384-Well Block Module (e.g., 7900HT, Applied Biosystems).
14. Real-Time PCR system software (e.g., SDS 2.4, Applied Biosystems).
15. Real-Time PCR analysis software (e.g., RQ manager 1.2.1, Applied Biosystems).

3 Methods

3.1 Immunization and Tissue Preparation

1. C57BL/6 mice are primed with 50 μ g CGG in alum precipitated with 2×10^7 heat inactivated *B. pertussis* bacteria as an adjuvant.
2. After 5 weeks, the mice are boosted with 50 μ g soluble NP₁₈-CGG in PBS, via intraperitoneal (*i.p.*) injection.
3. Termination of the response is from 5 days after immunization with soluble NP-CGG.
4. Spleens are removed, placed on a piece of aluminium foil, and snap frozen in liquid N₂.
5. After wrapping the frozen tissue in aluminium foil, spleens are stored at -80°C in grip-seal polythene bags until sections are cut (*see Note 2*).
6. The spleen is mounted on a cryostat holder using a small amount of OCT compound (*see Note 3*).
7. Trim off any excess of OCT compound (*see Note 4*).
8. Eight micrometers thick spleen sections are distributed equally onto a NF membrane slide (*see Note 5*). Approximately every 10 cuts, 1 or 2 sections (6 μ m) are mounted onto 4-spot glass slides in order to perform immunohistochemistry of these tissues. These will allow the identification of the different regions in regard to the adjacent tissue sections on the membrane slides.

9. Membrane and 4-spot glass slides are air-dried under a fan for 30 min then fixed in fresh analytical-grade acetone at 4 °C for 20 min, air-dried for 10 min (*see Note 6*).
10. Glass slides are stored in sealed polyethylene bags at –20 °C until use. Membrane slides are stored in –80 °C in the original manufacturer's storage box.

3.2 Immuno-histological Staining of Glass Slides

The 4-spot glass slides are stained by immunohistochemistry to identify different splenic regions (Fig. 1a, b). The example described here works well to differentiate white pulp into T zones, follicles, germinal centers, and plasma cell areas. Particularly GCs cannot be differentiated from follicles by cresyl violet staining

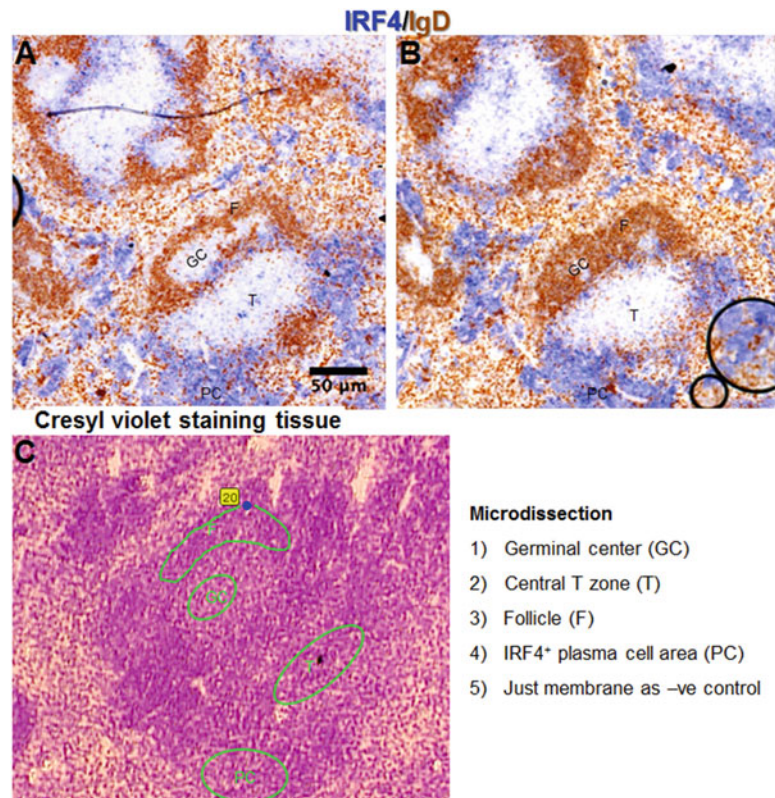


Fig. 1 Appearance of the sections from a consecutive series of tissue sections, stained by immunohistochemistry for IgD and IRF4, or with cresyl violet. Photomicrographs are from a spleen 5 days after NP-CGG immunization of a carrier-primed mouse. (a, b) For orientation, every tenth serial section is stained by immunohistochemistry for IgD (*brown*, B cell follicles) and IRF4 (*blue*, plasma cells). Scale bar: 50 μm. (c) A section from the same series stained with cresyl violet, showing the white pulp area in the bottom right of a, and b. This shows intense purple staining of the B cell follicles and plasma cell areas, slightly weaker staining of the GC and T zone, and light staining of the red pulp

alone. Optimum dilutions of all reagents are predetermined to give clear positive staining with minimum background. Spleen sections are stained with IgD and IRF4. Germinal centers are IgD negative areas within follicles, central areas containing central arterioles are T zone. IRF4 stains plasmablasts and plasma cells. A detailed staining protocol has been described before [5].

1. Defrost microscope slides in unopened polyethylene bags for 10 min under a fan.
2. Rehydrate slides by immersing in Tris buffer (pH 7.6) for 5 min in a wash trough at room temperature.
3. Rehydrated sections are covered with diluted primary antibodies: Goat anti-mouse IRF4, rat anti-mouse IgD. Incubate slides for 1 h at room temperature in a humid chamber.
4. At the end of the first incubation, slides are washed twice in Tris buffer for 5 min, using a wash trough and a magnetic stirrer.
5. Secondary antibodies are pre-absorbed 30 min before use in 10% normal mouse serum to eliminate anti-mouse immunoglobulin cross-reactivity. The secondary antibodies are biotinylated donkey anti-goat biotin, and horseradish peroxidase (PX) rabbit anti-rat.
6. Secondary antibodies are added to the sections and left to react for 45 min. After this excess antibodies are washed off in Tris buffer (pH 7.6) for 5 min.
7. Streptavidin alkaline phosphatase (AP) complex is added for biotinylated secondary antibodies. Streptavidin AP complex is prepared 30 min before use, added onto section and left to react for 30 min.
8. After washing, stains are developed using first the PX substrate solution for PX conjugated to secondary antibodies, and then AP substrate solution for biotinylated antibodies. Substrate solutions have to be made fresh before use, and wash for 5 min between adding the two substrate solutions. Developed slides are washed twice in distilled H₂O (dH₂O) and then mounted using glycerol gelatin and coverslips.
9. Scan the stained slides with a slide scanner, e.g., a Zeiss AxioScan Z1. Print the scanned images for reference to help identifying different areas on membrane slide when performing microdissection.

3.3 Cresyl Violet Staining of Membrane Slides

An ethanol-based cresyl violet staining is used for membrane slides. The use of an ethanol based dye provides maximal protection of RNA from degradation (Figs. 1c and 3) [6].

1. Dissolve 0.1 g of cresyl violet acetate in 10 ml of 75% ethanol (molecular biology grade) (1% W/V).

2. Leave in the dark at room temperature, rotate overnight to dissolve completely and then filter the next day.
3. Make 100% ethanol, 70% ethanol in water (aqua ad iniectabilia, pH balanced around 7.0) (*see Note 7*).
4. Defrost the membrane slides in the unopened container for 10 min under a fan.
5. Rehydrate the membrane slide in 70% ethanol; just leave the slide in the tube for seconds and not for longer than 30 s.
6. Leave the slide in a chamber, incubate for 1–2 min with cresyl violet solution (*see Note 8*) and then tip off the remaining liquid from the slide.
7. Dehydrate the slide via subsequent washes in 70%, and 100% ethanol.
8. Leave the membrane slide to air dry. The slide is ready for microdissection immediately after staining and can also be stored at -20°C until use.
9. PALM Liquid Cover Glass N (Zeiss) is used to improve the visible details of tissue sections before microdissection. The working solution is prepared according to manufacturer's instructions (*see Note 9*).

3.4 Microdissection of Different Areas on the Spleen Section

White pulp (dark areas) and red pulp (light areas) can be identified on sections stained with cresyl violet (Fig. 1). The location of B cell follicles, germinal centers, T zones and plasma cell areas can be confirmed by checking the printed images of the sections immunohistochemically stained with IRF4 and IgD. A microdissection experiment may include the identification of GC (IgD negative area), follicle (IgD positive), T zone, and plasma-blast/plasma cell rich extrafollicular foci (IRF4 positive) (*see Note 10*).

1. A Microbeam HT microscope with software Palm@robo V3.0 (Zeiss) is used (Fig. 2). The slides are mounted in the slide holder with the tissue section facing upwards. Initially an accurate overlap between the pointer in the Palm Robo software and the actual cutting position needs to be made (Fig. 3a, b). Then, the laser power for cutting and laser pressure catapulting (LPC) are adjusted (Fig. 3) (*see Note 11*). The settings are saved for future use. Cutting efficiency may be inhibited by OCT compound (*see Note 4*).
2. Identify the same white pulp area in the adjacent sections on the membrane slides (Fig. 1).
3. Prepare 8-well cap strips containing 20 μl of RNeasy RLT buffer. Make sure the buffer covers the cap completely.

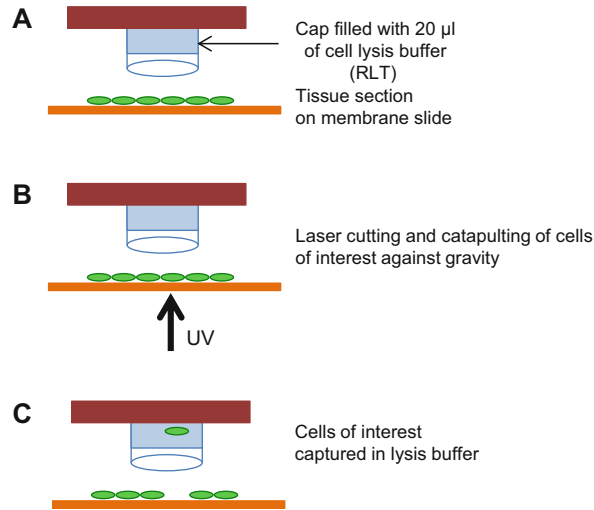


Fig. 2 Mechanism of tissue laser capture using the PALM microscope. (a) When isolating cells with the PALM–LCM system, tissue sections are firstly prepared on membrane-coated microscope slides and collecting caps are filled with 20 μ l RLT lysis buffer. (b) The UV laser is focused onto the section and used to cut around the area of interest. The laser then automatically focuses below the area of interest and a single stronger laser pulse is fired. (c) Catapulting of tissue into the overlying microcentrifuge tube cap

4. In order to get a good RT-PCR signal, normally 10–20 similar areas are selected for adjacent sections for each of the areas identified and catapulted into the same cap (Fig. 1a, b). Catapult different areas into separate caps (*see* **Note 12**).
5. After collecting the tissue clip the 8-well strips of caps onto 200 μ l RNase-free 96-well PCR plates.
6. Cut membrane only (tissue free area) into separate caps as a negative control for RNA contamination after finishing each type of area (*see* **Notes 13** and **14**) (Fig. 4).
7. Spin down by high speed, leave on dry ice, and then store in -20°C freezer until use.

3.5 RNA Isolation

It is important to extract and isolate RNA from the microdissected samples prior to qRT-PCR analysis to reduce the failure rate of qRT-PCR.

1. RNA is isolated by using the RNeasy Micro kit (*see* **Note 15**). RNA carrier in the kit is used to enhance RNA extraction from the microdissected tissue. For tubes with less than 5000 cells, 20 ng of carrier RNA (5 μ l of a 4 ng/ μ l solution) are added to the lysate before homogenization (*see* **Note 16**).



Fig. 3 Identification and microdissection of an identified area from a spleen section. (a) Follicular area identified and marked. (b) After laser cut, a small bridge at the top still connects the area to the rest of the section. (c) After laser pressure catapulting



Fig. 4 Microdissection of membrane only Identification and microdissection of tissue-free area (membrane only), as a negative control

2. Prepare an appropriate volume of buffer containing 10% β 2-mercaptoethanol (β -ME) in RLT buffer plus RNA carrier. Always make this buffer fresh (*see Note 17*).
3. Prepare an appropriate volume of 70% and 80% ethanol in RNase-free water (*see Note 18*).
4. Take samples out of the freezer and leave on dry ice until use. To minimize RNA degradation, avoid the prolonged exposure of samples to room temperature.
5. Add 150 μ l of RLT buffer to the sample in a 200 μ l PCR tube before the tissue is completely thawed. Mix well by pipetting. Transfer the sample and RLT buffer into a 1.5 ml microcentrifuge tube, adjust the sample volume to 350 μ l with buffer RLT and mix very well. Finally, transfer the total volume into a QIAshredder column.
6. Follow the kit instructions for the subsequent steps. During the RNA extraction, DNase treatment for 15 min is recommended.
7. The protocol ends with the RNA elution. Add just 10–15 μ l of RNA free water into the RNeasy column in order to increase the RNA concentration. If cDNA can't be prepared immediately then store the RNA at -80°C for the required period (*see Note 19*).

3.6 cDNA Preparation

1. Prepare a sufficient number of 0.2 ml 8-well strips of PCR tubes.
2. 10 μ l of RNA solution is mixed with 1 μ l of random primers (0.5 μ g/ μ l).
3. Denature the samples for 10 min at 70 °C in the thermocycler. Place on ice immediately after the 10 min.
4. 9 μ l of reverse transcription mix are added and mixed well. The following reagents are added to each sample: 0.5 μ l dH₂O (RNA free water); 4 μ l 5 \times first strand buffer; 2 μ l 0.1 M DTT; 1 μ l dNTP (10 Mm) (diluted from 100 mM set); 0.5 μ l RNase Inhibitor (40 μ g/ μ l); 1 μ l Moloney murine leukemia virus (M-MLV) reverse transcriptase.
5. Take the samples back to thermocycler for the reverse transcription of RNA into cDNA. Heat for 1 h at 41 °C, and then for 10 min at 90 °C to inactivate reverse transcriptase.
6. The cDNA is stored at -20 °C.

3.7 Semiquantitative Real Time PCR (qRT-PCR)

qRT-PCR is used to quantify the expression of genes of interest such as immunoglobulin heavy chain germ line transcripts, chemokines, or cytokines in cDNA prepared from microdissected tissue [7, 8].

1. PCR is performed by adding 1 μ l of cDNA to each well of a 384 well plate followed by the required primers and FAM-labeled probe mixtures and 3 μ l Universal qRT-PCR master Mix (final volume is 6 μ l) (*see Notes 20–22*).
2. Cover the plate with clear adhesive foil, and centrifuged to remove any air bubbles (689 \times g, a few seconds). Along with the amplification of the targeted gene, the internal housekeeping gene β 2-microglobulin is amplified so that the relative quantities of gene amplification can be assessed regardless of the number of cells harvested to produce each cDNA sample (*see Note 23*).
3. Run plate in an Real-Time PCR System with 384-Well Block Module with a cycling program as follows: 2 min at 50 °C; 10 min at 95 °C; Then 40 cycles of 15 s at 95 °C and final step of 1 min 60 °C.
4. Analyze data using an appropriate software, and set a threshold within the logarithmic phase of the PCR. The cycle number (Ct) at which the signals for target gene and housekeeping gene reach the threshold are recorded for each sample. Assuming gene of interest and the housekeeping gene amplify with similar efficiency, the relative quantity of expressed target gene mRNA is estimated by subtracting the Ct for the housekeeping gene from the Ct of the target gene (Δ Ct) and then calculating $2^{-\Delta$ Ct (Fig. 5).

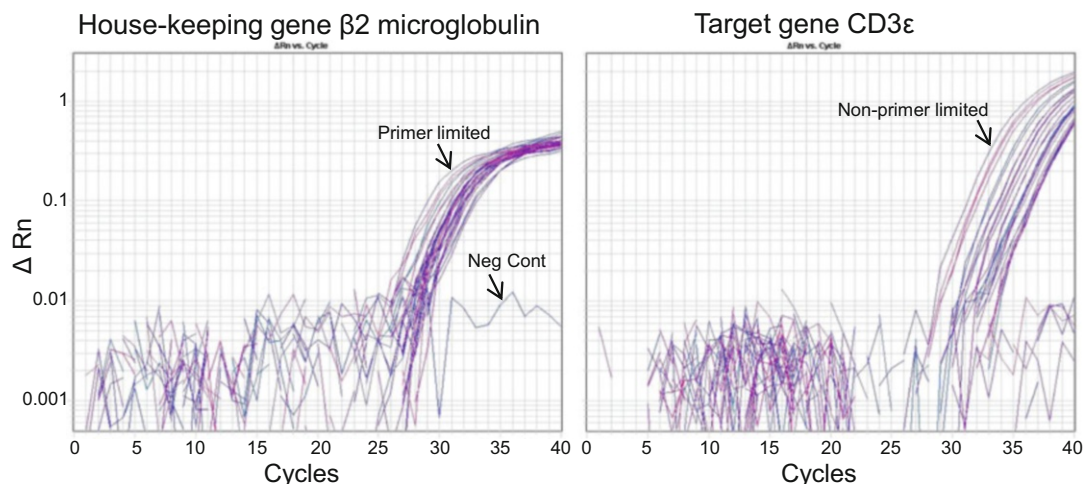


Fig. 5 Typical TaqMan qPCR amplification plots from 384-well setup from microdissected tissue. Background subtracted fluorescence signal (ΔRn) versus cycle number. Primer/probe concentrations for TaqMan PCR are usually optimized for maximal ΔRn and lowest Ct values. *Left*: The housekeeping gene is amplified using primer limiting conditions and a VIC-labeled probe. Limiting primers specific for the stronger expressed housekeeping gene prevents the PCR being depleted of nucleotides before the target gene specific fluorescence becomes detectable. *Right*: Genes of interest are measured using FAM-labeled non-primer limited primer/probe sets. Triplicate technical replicates result in good reproducibility. Neg Cont: Negative control (microdissected membrane only)

4 Notes

1. Chicken gamma globulin (CGG) is precipitated in alum (detailed protocol in Toellner et al. 2004). Briefly, CGG is made up as a 5 mg/ml solution in sterile water and added to an equal volume of sterile 9% $AlK(SO_4)_2$ in water. The protein/alum mix is precipitated with 10 M NaOH to pH 6.5, and left in dark for 1 h at room temperature, rotating to allow for maximum precipitation. Important: NP-CGG alum is precipitated with 10 M NaOH to pH 6.5 and 0.1 N HCl. If the pH is too high (e.g., pH 10.0), just discard the mixture and restart again. The precipitate is washed twice in sterile PBS. The pellet is suspended to a final concentration of 50 $\mu g/200 \mu l$ in sterile PBS with 5×10^7 heat inactivated *Bordetella pertussis* (b.p.) bacteria as an adjuvant for intraperitoneal (i.p.) injection. The preparation of 4-hydroxy-3-nitrophenylacetyl (NP)-CGG has been described before [5, 9]. NP-O-succinimide ester (NP-OSu) is dissolved at 10 mg/ml in dimethylformamide (DMF). CGG is dissolved at 10 mg/ml in 0.1 M sterile $NaHCO_3$ on ice. NP-OSu is added drop by drop, while stirring, into the protein solution at a ratio of 1 mg NP: 20 mg protein and then incubated at room temperature in the dark for 2 h on a rotating mixer. The NP-Protein solution is dialyzed against 0.1 M $NaHCO_3$ overnight and then against sterile PBS (pH 7.4)

overnight using dialysis cassettes (10,000 MW). This step removes any non-conjugated NP from the mixture. This procedure gives an approximate substitution rate of 18 NP per molecule of protein.

2. Termination of response is at day 5, or day 8, depending on the experiment. Freeze tissue samples slowly by repeated dipping in liquid N₂. Snap-frozen samples provide the highest quality of DNA, mRNA, and protein for analysis [10]. Do not store the tissue in the freezer for too long, even at -80 °C.
3. Wear gloves and a laboratory coat. Put a small strip of OCT compound on the cold cutting block and place the spleen on top, freeze immediately using freeze spray. Trim the spleen a little bit until the white pulp is visible, before starting to mount sections onto microscope slides.
4. Make sure any OCT compound that may come into contact with the microtome knife is carefully trimmed off before cutting. OCT can seriously inhibit microdissection efficiency by interfering with the microdissection laser light.
5. If the tissue does not stick well to membrane slides, warm the membrane slides by putting a finger on the back of the slide when mounting the section from the cryostat knife. Then dry the slide at room temperature under a fan.
6. Fix membrane slides and normal glass slides in separate glass troughs. Especially membrane slides should be fixed in a RNase/DNase-free glass trough.
7. For cresyl violet staining, make up 100% ethanol and 70% ethanol in water solutions in separate 50 ml FALCON tubes. Then just soak the slides in these tubes for seconds. Here *aqua ad iniectabilia* is used (pH 7.0). The pH value may affect the quality of cresyl violet staining and RNA extraction [6].
8. The timing for cresyl violet staining should be optimized using spare tissue sections. Normally for murine lymph node and spleen, 1–2 min are sufficient, while murine kidney stains well within a few seconds. Dependent on the tissue cresyl violet in 50% ethanol in water may give better results.
9. Make a working solution in a spray bottle as a stock solution to thinning solution in a ratio of 1:6. Spray once at a distance of 10–20 cm onto the membrane slides and allow to dry for approximately 5 min. For spleen and lymph node sections, the manufacturer's recommended concentration works well. Other types of tissues may need optimization with a different dilution of stock solution or repeated spaying and drying cycles.
10. Have separate pipettes for performing microdissection and cDNA preparation. Clean the pipettes, working area, slide holder, collection holder of microdissection microscopy with RNaseZap® RNase Decontamination Solution (Ambion, Thermo Fisher).

11. Microscope is set to best focus with minimal cutting power, which should result in minimal tissue damage. Adjust the collection tube holder to make sure the microdissected tissue can be laser pressure catapulted into the cap or PCR tube. Use the Robo LPC mode for cutting and shooting tissue. Robo LPC mode cuts around the selected tissue area, leaving a small bridge which is destroyed during the subsequent laser pressure catapulting step. Set the right bridge width for optimal catapulting of the microdissected area. If the bridge is too big, the selected area may not catapult well and stick to the tissue. If the bridge is too narrow, the dissected section may drop off during cutting. *See also* **Note 4**.
12. Marking and microdissection of all corresponding areas in all adjacent sections will create easily identifiable landmarks to the next series of areas of interest.
13. If the tissue does not catapult into the cap, cut once more with same laser power. *See also* **Note 4**. Clean the membrane slides from residual contaminating tissue particles by placing under a fan for seconds before microdissecting other areas.
14. Cut one tissue section, do the cDNA prep, and use qRT-PCR to check whether the cut area is correct and one can get an appropriate amplification signal.
15. Normally, keep the higher concentration stock (400 µg/ml) in freezer (−20 °C). Dissolve RNA carrier at higher concentration solution (400 µg/ml) by using RNase-free water, and then save at −20 °C until use.
16. β-ME is toxic, so dispense in a fume hood and wear appropriate protective clothing. It is advisable to work in a fume hood when using buffer containing β-ME.
17. The RNase-free technique needs to be followed stringently to prevent contamination. Wear gloves, a clean laboratory coat and change gloves often. Clean the bench tops and pipettes with RNaseZap® RNase Decontamination Solution. Ideally a dedicated working area is required.
18. Here β2-microglobulin is chosen as the only housekeeping gene. If performing multiplex reactions, it is recommended to use primer-limited primer/probe sets for the amplification of the endogenous control gene (Fig. 5).
19. TaqMan® chemistry based primer/probe sets are chosen in our experiment. Primer/probe combinations are strongly recommended for use with RNA from laser microdissected samples because of the improved specificity over primer only based detection chemistries (e.g., SYBR green detection) [10]. Primer and probe design has been discussed extensively in other papers, for example [11].

20. In our experience the lowest volume that reliably produces efficient qRT-PCR amplification in 384-well plates is 1 μ l of cDNA with 5 μ l TaqMan mix. These volumes are also easily pipetted with common electronic multichannel pipettes.
21. After covering the plate with adhesive sealing film, shake the plate on a vortex mixer with a plate adapter to mix the cDNA with the reaction buffer and then centrifuge at a high speed for seconds. Centrifugation after shaking is essential for successful RT-PCR.
22. Always use cDNA extracted from whole spleen or lymph node sections as a positive control and RNase-free water as negative control for qRT-PCR. CD3 ϵ and CD19 are chosen as positive controls for successful dissection of different areas prior to testing the expression of other genes (Fig. 6).
23. We use a probe for β 2-microglobulin either VIC or NED labeled (e.g., Applied Biosystems) so as not to interfere with the FAM-labeled.

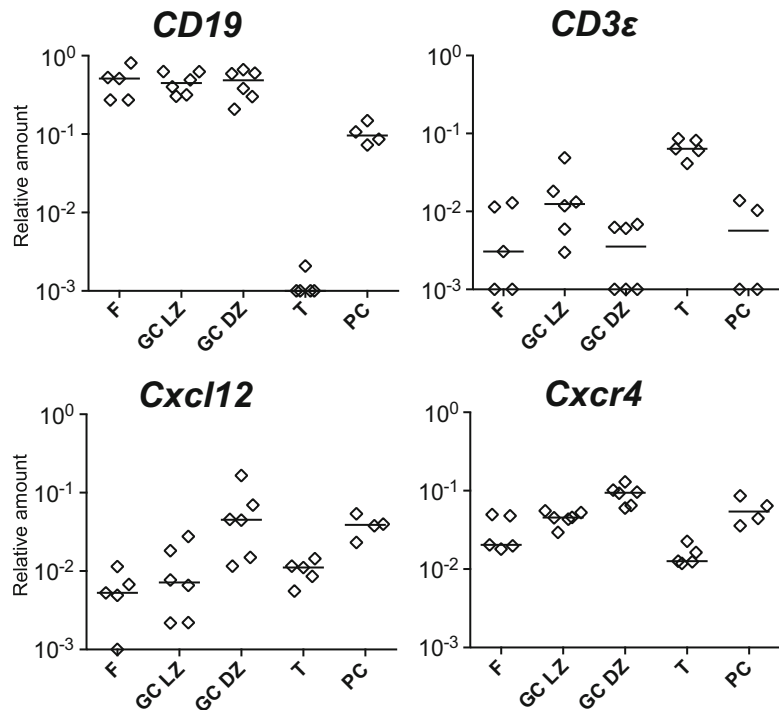


Fig. 6 Validation of qRT-PCR method from different areas of a laser microdissected spleen section. Relative expression levels for *Cd3 ϵ* , *Cd19*, *Cxcl12*, and *Cxcr4* from different microdissected areas. Expression levels are given as the amount of mRNA relative to the level of β 2m mRNA. F Follicle, GC LZ Germinal center light zone, GC DZ Germinal center dark zone, T T zone, PC IRF4⁺ Plasma cell area. Each diamond represents the pooled microdissected material from ten consecutive sections from one identified area

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Competing Financial Interests

The authors declare no competing financial interests.

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