Published in final edited form as:

J Immunol Methods. 2017 March ; 442: 59–63. doi:10.1016/j.jim.2017.01.003.

A Method for Obtaining Simian Immunodeficiency Virus RNA Sequences from Laser Capture Microdissected and Immune Captured CD68+ and CD163+ Macrophages from Frozen Tissue Sections of Bone Marrow and Brain

Jaclyn Mallard¹, Emily Papazian¹, Caroline Soulas^{1,2}, David J Nolan^{3,4}, Marco Salemi^{3,4}, and Kenneth C Williams^{1,*}

¹Department of Biology, Boston College, Chestnut Hill, MA, USA

²Department of Research and Development, Innate Pharma, Marseille, France

³Emerging Pathogens Institute, University of Florida, Gainesville, FL, USA

⁴Department of Pathology Immunology and Laboratory Medicine, University of Florida, College of Medicine, Gainesville, FL, USA

Abstract

Laser Capture Microdissection (LCM) is used to extract cells or tissue regions for analysis of RNA, DNA or protein. Several methods of LCM are established for different applications, but a protocol for consistently obtaining lentiviral RNA from LCM captured immune cell populations is not described. Obtaining optimal viral RNA for analysis of viral genes from immune-captured cells using immunohistochemistry (IHC) and LCM is challenging. IHC protocols have long antibody incubation times that increase risk of RNA degradation. But, immune capture of specific cell populations like macrophages without staining for virus can result in obtaining only a fraction of cells which are productively lentivirally infected. In this study we sought to obtain simian immunodeficiency virus (SIV) RNA from SIV gp120+ and CD68+ monocyte/macrophages in bone marrow (BM) and CD163+ perivascular macrophages in brain of SIV-infected rhesus macaques. Here, we report an IHC protocol with RNase inhibitors that consistently results in optimal quantity and yield of lentiviral RNA from LCM-captured immune cells.

*Direct requests for reprints and other inquiries to: Professor Kenneth C Williams, Ph. D., Department of Biology, Boston College, Higgins Hall, Room 468, 140 Commonwealth Avenue, Chestnut Hill, MA 02467, kenneth.williams.3@bc.edu.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

Author Contributions

J.M. did the experimental design and optimization of immuno-staining, LCM and RNA isolation with help from C.S. and D.J.N. J.M. and E.P. acquired viral RNA from LCM-captured macrophages and performed cDNA synthesis and PCR analysis. M.S. contributed to the experimental conception of viral sequence analysis. K.C.W. formulated the idea to optimize an LCM protocol for obtaining lentiviral RNA from macrophages for assessment of viral evolution. K.C.W. and J.M. interpreted the results and wrote the manuscript.

Competing Interests Statement

The authors declare no competing interests.

Keywords

Laser Capture Microdissection (LCM); Immunohistochemistry (IHC); Simian Immunodeficiency Virus (SIV); RNA

2. Introduction

Analysis of viral reservoirs within specific tissues has implications for AIDS-related research of HIV-associated cardiovascular and neurocognitive disorders. Studying lentiviral RNA from cells in tissues can elucidate mechanisms of viral persistence and immune responses to infection. Lentiviral RNA isolated from immune-captured cells using laser capture microdissection (LCM) can provide insight into changes in viral sequences, genes and evolution within infected cell populations as they may relate to pathogenesis.

Several LCM protocols exist that use frozen or paraffin-embedded tissue sections [1–7]. These have been optimized for obtaining RNA from specific cell populations identified by immunohistochemistry then captured using LCM (immune-captured) or tissue regions. Each protocol employs rapid antibody staining (1–10 minute incubations) followed by LCM and RNA isolation from captured samples with a focus to minimize RNA degradation [2–6]. Obtaining sufficient yields and quality of lentiviral RNA for analysis requires rapid LCM methods and strict RNase-free conditions. Generally, capturing large regions of tissues increases the odds of obtaining sufficient quantities of RNA, thereby lessening the effects of RNase-mediated degradation. This approach is not adequate to obtain RNA from enriched, purified cell populations. The inability to obtain sufficient yields of viral RNA from immune-captured cells with LCM is due to: the use of antibodies for IHC that increase the total time for the staining procedure which heightens the risks for RNA degradation, and the fact that often only a fraction of captured cells are potentially infected.

Our studies focus on SIV-infection of immune captured myeloid cells from bone marrow (BM) and the central nervous system (CNS) to understand their role in carrying virus to the CNS and their functions as viral reservoirs. Protocols published to date obtain cellular RNA from captured cells [1–6]. None are optimized for obtaining SIV RNA from infected tissue macrophages. Matsuda *et al* published a methodology using LCM to isolate SIV-RNA from CNS tissue regions, but this study did not immune capture for individual cells [7]. To date, a method for LCM of immune captured cells to obtain sufficient quality and yield of lentiviral RNA for cDNA amplification is not described. We have optimized a method for LCM of immuno-stained cell populations from frozen tissue sections that yields sufficient quantities and quality of viral RNA for cDNA synthesis and PCR amplification. Using this method, we consistently amplify SIV cDNA from RNA isolated from immuno-stained macrophage populations captured from frozen BM and brain tissue sections from SIV-infected rhesus macaques.

3. Theory

A method for obtaining lentiviral RNA from LCM-captured immune captured CD68+ and CD163+ macrophages allows assessment of viral infection within enriched cell populations

as it may relate to lentiviral pathogenesis. Analysis of lentiviral RNA within infected cell populations allows assessment of viral sequence evolution. Phylogenetic analysis of viral sequences can be used to infer viral migration patterns throughout the course of infection and to assess compartmentalization of virus and identify the source of viral reservoirs.

4. Materials and Methods

4.1 Ethics statement

There were no live animals used in this study. We analyzed archival tissues from SIV-infected rhesus macaques from previous studies. The initial studies were approved by and strictly complied with the guidelines for animal care as defined by the Institutional Animal Care and Use Committees at the Tulane National Primate Research Center and Harvard University's New England Regional Primate Center.

4.2 Animal Tissues

Tissue samples from BM (n = 10) and CNS cortices (frontal, occipital, parietal and temporal) (n = 3) were collected from SIV-infected rhesus macaques at necropsy, embedded in optimal cutting medium (OCT) and stored at -80°C . Prior to LCM, frozen tissue sections were warmed to -20°C , cut into $8\text{ }\mu\text{m}$ sections using a cryostat (Leica, Buffalo Grove, IL, USA, Model #CM3050 S), and mounted on membrane-free LCM glass slides (Histogene LCM Frozen Section Staining Kit, Applied Biosystems, Carlsbad, CA, USA Cat. #KIT0401) that were treated with RNaseZap (Life Technologies, Carlsbad, CA, USA, Cat. #AM9782). Sections were immediately placed on dry ice, wrapped in tin foil and stored at -80°C .

4.3 Immunohistochemistry for LCM

Frozen tissue sections were removed from -80°C and thawed in slide jars containing RNALater (Qiagen, Valencia, CA, USA) at room temperature (RT). A de-humidified (<50% humidity), RNase-free room was used for IHC, LCM, RNA isolation, cDNA synthesis and PCR amplification. Immediately prior to IHC, slide jars were cleaned with 75% ethanol and nuclease-free water then sprayed with RNaseZap. RNA Protector (Roche, Indianapolis, IN, USA) was added to primary and secondary antibodies ($0.4\text{ U}/\mu\text{L}$) and the DAB chromagen substrate solution. Primary antibodies; mouse monoclonal SIV mac251 p28 (Fitzgerald Industries, Acton, MA, USA, clone 3F7) ($5\text{ }\mu\text{g}/\text{mL}$), mouse anti-human CD68 (Dako, Carpinteria, CA, USA, clone KP1) ($8\text{ }\mu\text{g}/\text{mL}$) or mouse anti-human CD163 (Serotec, Raleigh, NC, USA, clone EDhu-1) ($40\text{ }\mu\text{g}/\text{mL}$) were diluted in $1\times\text{PBS}$. Thawed tissue sections were fixed in chilled acetone (20°C) for 3 minutes at RT then dipped in $1\times\text{PBS}$ and encircled using a wax pen (Dako). Tissues were incubated with the primary antibody for 2 minutes (SIV p28 or CD68) or 8 minutes (CD163) at RT. Tissues were washed twice in $1\times\text{PBS}$ to remove antibodies then incubated with anti-mouse polymer HRP (Dako) for 2 minutes at RT and washed twice in $1\times\text{PBS}$. The peroxidase reaction product was developed with DAB chromagen (Dako) for 30 seconds at RT then stopped in nuclease-free water. Tissues were dehydrated using graded ethanols and then cleared with xylenes using the Histogene LCM Frozen Section Staining Kit (Applied Biosystems). Slides were dried in a container with silica desiccant (Sigma) then immediately used for LCM.

4.4 Laser Capture Microdissection

Immuno-stained cell populations; SIV p28+ virally infected cells and CD68+ macrophages in BM and CD163+ CNS perivascular macrophages were laser microdissected using an ArcturusXT™ LCM System and operating software (Applied Biosystems). A capture HS polymer cap (Applied Biosystems) was placed on a tissue region containing immuno-stained cells and a minimum of 400 cells were captured. Infrared (IR) laser shots (18–20 µm diameter) were fired at 70 mV, capturing cells onto the polymer of the HS capture cap. The time between immuno-staining and IR capture was kept to a maximum of 30 minutes to minimize RNA degradation.

4.5 RNA Isolation

Capture HS polymers with LCM captured cells were peeled off of the HS capture cap using RNase-free forceps. Caps were submerged into an RNase-free microtube containing 50 µL of Extraction Buffer (Pico Pure RNA Isolation Kit, Applied Biosystems) and incubated at 42°C for 30 minutes in a thermocycler as this has been reported to increase RNA yields [2]. Total RNA was isolated using the Pico Pure RNA Isolation Kit (Applied Biosystems). Concentrations of mRNA from LCM-captured CD68+ and CD163+ macrophages were determined using the mRNA Pico analysis assay on the Agilent Bioanalyzer 2100. LCM of 400 immune-captured cells yielded mRNA concentrations between 50–100 pg/µL.

4.6 cDNA Synthesis and PCR-amplification

Total RNA isolated from captured cells was converted to cDNA using the Superscript III First Strand Synthesis Kit (Life Technologies) with the Oliog-dt RNA primer per the manufacturer's instructions. Reverse transcription was performed at 45°C for 2 hours. Control reactions were performed to confirm that captured cells contained CD68 cDNA (BM) or CD163 cDNA (brain) and were not contaminated with T cells (CD3). This was done using Platinum Blue PCR mix (Life Technologies) per the manufacturer's protocol with primers for CD3, CD68 or CD163 (Table 1). The remaining cDNA was amplified using Platinum blue PCR mix (Life Technologies) with primers directed toward SIV envelope gp120 cDNA (SIV *gp120*) (Table 1). This was done in two rounds of nested PCR using parameters previously optimized for SIV *gp120* cDNA amplification; 94 °C for 15 minutes, 40 cycles of 94 °C for 15 seconds, 50 °C for 30 seconds, 68 °C for 6 minutes followed by a final cycle at 68 °C for 6 minutes [8]. Primers used for the first round of nested PCR targeted the SIV *gp120* amplicon between 6565 – 8205 bp (SOUT). The PCR product from the first round was used as the template for the second round of nested PCR with primers that targeted the SIV *gp120* amplicon 6598–8184 bp (SIN). Amplified SIV *gp120* cDNA (1.5 kb) and control cDNA (CD3 (500 bp), CD68 (200 bp), and CD163 (400 bp)) were visualized with ethidium bromide on 0.7% (SIV *gp120*) and 1.7% agarose gels, respectively. The concentration of amplified SIV *gp120* cDNA was determined using a Typhoon FLA 9500 laser scanner (GE Healthcare, Pittsburgh, PA, USA) and ImageQuant TL 8.1 image analysis software (GE Healthcare).

5. Results and Discussion

5.1 SIV *gp120* cDNA amplified from SIV-RNA of LCM SIV p28+ cells

As a control, we first sought to determine if there was detectable SIV RNA in bone marrow (BM). We isolated total RNA from unfractionated (without LCM) BM tissues from SIV-infected rhesus macaques ($n = 10$) and performed cDNA synthesis and amplification of SIV *gp120* cDNA using nested PCR (Figure 1A). SIV *gp120* cDNA was amplified from unfractionated BM from all animals ($n = 10$).

Next we attempted to generate SIV *gp120* cDNA from SIV-RNA of LCM-captured SIV p28+ cells in BM. To minimize the risk of RNA degradation: we thawed frozen tissue sections in RNALater (Qiagen), added an RNase inhibitor (0.4 U/ μ L) to the primary and secondary antibodies and the DAB substrate solution and used rapid antibody incubation times (2–8 minutes). Images documenting the staining distribution of BM sections before and after LCM of SIV p28+ cells confirmed successful capture of SIV p28+ cells (Fig. 1B). Total RNA was isolated from captured cells and cDNA was synthesized. Because we anticipated low quantities of SIV RNA, we set up 10 nested PCR reactions with our cDNA template to amplify SIV *gp120* cDNA. Amplification of SIV *gp120* cDNA was observed in 1 of the 10 reactions, confirming the low yield of SIV RNA from SIV p28+ LCM captured cells (Figure 1C).

5.2 Amplification of SIV *gp120* cDNA from SIV-RNA of CD68+ BM macrophages

Next we focused to amplify SIV *gp120* cDNA from immune-captured CD68+ BM macrophages. In our initial experiments we captured SIV p28+ cells; therefore we captured cells with a high probability of being SIV RNA+ (Figure 1). Still, the yield of SIV RNA from LCM captured cells was low. Cell populations in BM that can be targets of SIV infection include CD68+ macrophages and CD3+ T lymphocytes. SIV *gp120*, CD3 and CD68 cDNA was amplified from total RNA isolated from unfractionated BM that was not subjected to LCM from all animals studied ($n = 10$) (Figure 2). To amplify SIV *gp120* cDNA from BM macrophages, we captured 400 CD68+ macrophages from frozen BM sections, isolated total RNA and synthesized cDNA. RNA concentrations were determined using the Agilent Bioanalyzer 2100 RNA Pico Kit mRNA analysis assay and ranged from 50–100 pg/ μ L from BM CD68+--captured cells. SIV *gp120* cDNA was amplified from laser captured CD68+ BM macrophages from all animals studied ($n = 10$) (Figure 2A). Control PCR showed that CD68, but not CD3 cDNA was amplified from LCM-captured CD68+ BM macrophages (Figure 2B). These results demonstrated the purity of the laser captured CD68+ macrophages and the ability to obtain highly enriched CD68+ macrophages from frozen BM using LCM without contamination with T lymphocytes. Moreover, this confirmed by PCR that the source of the amplified SIV *gp120* cDNA following LCM was from captured CD68+ macrophages, not CD3+ T lymphocytes.

5.3 Amplification of SIV *gp120* cDNA from SIV-RNA of CD163+ CNS perivascular macrophages

SIV associated encephalitis (SIVE) is characterized by lesions in the CNS that are consequent of accumulation of infected and uninfected macrophages in chronic SIV

infection [12]. In this study, we aimed to obtain SIV *gp120* cDNA sequences from CD163+ CNS macrophages from animals that developed SIVE (n = 3). CD163+ CNS perivascular macrophages are known to have detectable SIV RNA and are a cell type that may carry virus into the CNS [9]. Similar to BM experiments, we amplified SIV *gp120* sequences from total RNA isolated from CD163+ CNS perivascular macrophages from all animals studied (n = 3) (Fig. 3A). RNA concentrations were determined using the Agilent Bioanalyzer 2100 RNA Pico Kit mRNA analysis assay and ranged from 50–100 pg/μL from CNS CD163+-captured cells. Total RNA isolated from control CNS tissue sections without LCM yielded cDNA amplification of CD3 and CD163 cDNA. However, total RNA from CD163+ LCM cells resulted in amplification of CD163, but not CD3 cDNA (Figure 3B). This confirmed that CD163+ CNS perivascular macrophages were the source of the identified SIV *gp120* cDNA sequences. These results demonstrated that our LCM protocol is a reliable and reproducible method for obtaining SIV *gp120* sequences from monocyte/macrophages that were identified by various immune markers from different tissues.

6. Conclusions

Here we describe a protocol for LCM that allows successful isolation of lentiviral RNA from immune-captured CD68+ and CD163+ infected macrophages in frozen sections of bone marrow and brain tissues. While several LCM protocols have been optimized for obtaining RNA from immune-captured cells, our method yields sufficient lentiviral RNA from enriched macrophages without staining for virus. This method allows analysis of lentiviral sequence evolution within infected cell populations to identify their role in lentiviral pathogenesis.

Acknowledgments

This work was supported by the National Institute of Neurological Disorders and Stroke [R01 NS040237]. This paper is subject to the NIH Public Access Policy.

References

1. Fend F, Emmert-Buck MR, Chuaqui R, Cole K, Lee J, Liotta LA, Raffield M. Immuno-LCM: Laser capture microdissection of immunostained frozen sections for mRNA analysis. *Am J Pathol.* 1999; 154:61–66. [PubMed: 9916919]
2. Gallup JM, Kawashima K, Lucero G, Ackermann MR. New quick method for isolating RNA from laser captured cells stained by immunofluorescent immunohistochemistry; RNA suitable for direct use in fluorogenic TaqMan one-step real-time RT-PCR. *Biol Proced Online.* 2005; 7:70–92. [PubMed: 16136226]
3. Keays KM, Owens GP, Ritchie AM, Gilden DH, Burgoon MP. Laser capture microdissection and single-cell RT-PCR without RNA purification. *J Immunol Methods.* 2005; 302:90–98. [PubMed: 16084216]
4. Xu C, Houck JR, Fan W, Wang P, Chen Y, Upton M, Futran ND, Schwartz SM, et al. Simultaneous isolation of DNA and RNA from the same cell population obtained by laser capture microdissection for genome and transcriptome profiling. *J Mol Diagn.* 2008; 10:129–134. [PubMed: 18258925]
5. Feig JE, Fisher EA. Laser capture microdissection for analysis of macrophage gene expression from atherosclerotic lesions. *Methods Mol Biol.* 2013; 1027:123–135. [PubMed: 23912984]
6. Kimura Y, Kubo S, Koda H, Shigemoto K, Sawabe M, Kitamura K. RNA analysis of inner ear cells from formalin fixed paraffin embedded (FFPE) archival human temporal bone section using laser microdissection--a technical report. *Hear Res.* 2013; 302:26–31. [PubMed: 23660400]

7. Matsuda K, Brown CR, Foley B, Goeken R, Whitted S, Dang Q, Wu F, Plishka R, et al. Laser capture microdissection assessment of virus compartmentalization in the central nervous systems of macaques infected with neurovirulent simian immunodeficiency virus. *J Virol.* 2013; 87:8896–8908. [PubMed: 23720733]
8. Hill A, Leviyang S, Newman R, Zody M, Yang X, Ita S, Mangano M, Kirmaier A, et al. Emergence of simian immunodeficiency virus in rhesus macaques after cross-species transmission is characterized by changes in structural and accessory genes that enhance fitness in the new host species. 2016 In preparation.
9. Soulas C, Conerly C, Kim WK, Burdo TH, Alvarez X, Lackner AA, Williams KC. Recently infiltrating MAC387(+) monocytes/macrophages: A third macrophage population involved in SIV and HIV encephalitic lesion formation. *Am J Pathol.* 2011; 178:2121–2135. [PubMed: 21514427]
10. Lamers SL, Nolan DJ, Rife BD, Fogel GB, McGrath MS, Burdo TH, Autissier P, Williams KC, et al. Tracking the Emergence of Host-Specific Simian Immunodeficiency Virus env and nef Populations Reveals nef Early Adaptation and Convergent Evolution in Brain of Naturally Progressing Rhesus Macaques. *J Virol.* 2015; 89:8484–8496. [PubMed: 26041280]

Highlights

- A method for obtaining lentiviral RNA from LCM-captured immune-selected CD68+ and CD163+ macrophages
- A modified method using rapid immunohistochemistry in the presence of RNase-inhibitors to optimize selection of lentiviral RNA from immune-selected CD68+ and CD163+ macrophages from frozen tissues

Figure 1. SIV *gp120* cDNA amplified from LCM SIV p28+ cells

A. SIV *gp120* cDNA amplification from unfractionated BM without LCM **B.** Representative image of a section of BM tissue that was IHC-stained for SIV p28+ cells (dark brown) before (top) and after (bottom) LCM captured cells. Red circles highlight SIV p28+ cells that were captured onto the HS capture polymer cap. **C.** SIV *gp120* cDNA (1.5 kb) amplified from 1000 SIV p28+ LCM-captured cells. Amplified SIV *gp120* cDNA (1.5 kb) was visualized using ethidium bromide on a 0.7% agarose gel.

Figure 2. Amplification of SIV *gp120* cDNA from CD68+ BM macrophages

SIV *gp120* was amplified from LCM-captured CD68+ macrophages from all animals studied (n = 10). **A.** Representative image of a 0.7% agarose gel showing SIV *gp120* cDNA amplified from unfractionated BM without LCM (left) and from 400 LCM-captured CD68+ BM macrophages (right). **B.** Representative image of a 1.7% agarose gel showing control PCR products from: **Left** - unfractionated BM without LCM (*left*) with amplification of both CD3 (500 bp) and CD68 (200 bp) cDNA and **Right** - LCM-captured CD68+ BM macrophages with amplification of only CD68 (200 bp) and not CD3 (500 bp) cDNA. LCM

of CD68+ cells from BM yielded 456 pg of RNA. The white arrow points to CD68 cDNA from LCM-captured CD68+ BM macrophages. Control (CD3 and CD68) and SIV *gp120* cDNA PCR products were visualized using ethidium bromide.

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

Figure 3. Amplification of SIV *gp120* cDNA from CD163+ CNS perivascular macrophages
 SIV *gp120* was amplified from LCM-captured CD163+ CNS perivascular macrophages from all animals studied (n = 3). **A.** Representative image of a 0.7% agarose gel showing SIV *gp120* cDNA amplified from unfractionated CNS cortices without LCM (left) and from 400 LCM-captured CD163+ CNS perivascular macrophages (right). **B.** Representative image of a 1.7% agarose gel showing control PCR products from: **Left** - unfractionated CNS cortices without LCM (**left**) with amplification of CD3 (500 bp), CD68 (200 bp) and CD163 (400 bp) cDNA and **Right** - LCM-captured CD163+ CNS perivascular macrophages with

amplification of only CD163 (400 bp) and not CD3 (500 bp) or CD68 (200 bp) cDNA. LCM of CD163+ cells from brain yielded 632 pg of RNA. The black arrow points to CD163 cDNA from LCM-captured CD163+ CNS perivascular macrophages. Control (CD3, CD68 and CD163) and SIV *gp120* cDNA PCR products were visualized using ethidium bromide.

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

Table 1
Oligonucleotide Sequences of Primers for cDNA amplification

Primer sequences for CD3, CD68 and CD163 were determined from the respective NCBI reference sequences listed for *Macaca mulatta*. Sequences for the 2-step nested PCR for SIV gp120 were determined as previously described.¹⁰

cDNA	Primer	Primer Sequence (5' → 3')
CD3	Forward	CACTCGCTGGAGAGTTCTG
	Reverse	GGTGGCCTCTCCTTGTTT
CD68	Forward	GGAATGACTGTCCTCACAAA
	Reverse	TTTCTGTGGCTGGTGGTG
CD163	Forward	CAAGGAGGATGCAGGAGTTATC
	Reverse	GTGGGTCCTTCTTGTAGTCTTATC
SIV gp120 SOUT (1 st Round Nested PCR)	Forward	GGCTAAGGCTAATACATCTTCTGCATC
	Reverse	ACCCAAGAACCCTAGCACAAAGACCCC
SIV gp120 SIN (2 nd Round Nested PCR)	Forward	GTAAGTATGGGATGTCTTGGGAATCAG
	Reverse	GACCCCTCTTTTATTTCTTGAGGTGCC