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IF-LCM: Laser capture microdissection of immunofluorescently defined cells for mRNA analysis

Rapid Communication

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IF-LCM: Laser capture microdissection of immunofluorescently defined cells for mRNA analysis.

Background. The next phase of the molecular revolution will bring functional genomics down to the level of individual cells in a tissue. Laser capture microdissection (LCM) coupled with reverse transcription-polymerase chain reaction (RT-PCR) can measure gene expression in normal, cancerous, injured, or fibrotic tissue. Nevertheless, targeting of specific cells may be difficult using routine morphologic stains. Immunohistochemistry can identify cells with specific antigens; however, exposure to aqueous solutions destroys 99% of the mRNA. Consequently, there is an overwhelming need to identify specific tissue cells for LCM without mRNA loss. We report on a rapid immunofluorescent LCM (IF-LCM) procedure that allows targeted analysis of gene expression.

Methods. A LCM microscope was outfitted for epifluorescence and light level video microscopy. Heat filters were added to shield the image intensifier from the laser. Frozen sections were fluorescently labeled by a rapid one minute incubation with anti–Tamm-Horsfall antibody and an ALEXA-linked secondary antibody. Fluorescently labeled thick ascending limb (TAL) cells were detected by low light level video microscopy, captured by LCM, and mRNA was analyzed by RT-PCR for basic amino acid transporter, Tamm-Horsfall protein, and aquaporin-2.

Results. The immunofluorescently identified TAL could be cleanly microdissected without contamination from surrounding tubules. The recovery of RNA following rapid immunofluorescence staining was similar to that obtained following hematoxylin and eosin staining, as assessed by RT-PCR for malate dehydrogenase.

Conclusions. We conclude that the new apparatus and method for the immunofluorescent labeling of tissue cells targeted for LCM can isolate pure populations of targeted cells from a sea of surrounding cells with highly acceptable preservation of mRNA. Since the TAL is minimally injured following ischemia, identification of the different responses between TAL and surrounding tissue in damaged kidneys may provide new therapeutic targets or agents for the treatment of acute renal failure.

Key words: microdissection, immunofluorescence, thick ascending limb, acridine orange, RNA preservation, video-microscopy.

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Study of gene expression at the mRNA level in anatomically complex organs is difficult because the total organ mRNA is "contaminated" by admixture of many different cell types. This problem has been greatly lessened by the advent of laser capture microdissection (LCM), which can isolate pure populations of defined cells from stained tissue sections [1–4]. The resulting mRNA can be amplified by reverse transcription-polymerase chain reaction (RT-PCR). LCM has been used to purify difficult to isolate populations of cells from cancers, small and large neurons, and renal glomeruli and tubules [2, 5–7]. The tissue obtained can be used to measure mRNA abundance of individual genes, generate expression libraries, perform subtractive hybridization cloning, or screen high density cDNA arrays [2, 7, 8]. LCM is especially useful for isolating structures from injured or fibrotic tissue since manual dissection under these conditions is impossible [6].

Most published reports have used LCM to isolate cells based on morphologic (cell size) or histologic staining characteristics. For example, differentiation of cancer versus normal cells or small versus large neurons is easy using light microscopy [2, 7]. However, many cells cannot be differentiated by histologic criteria. Differentiation of cells in mixed populations of tumors or structurally heterogenous organs is often difficult in paraffin-embedded sections and may be impossible in frozen sections. We found that identification of injured nephron segments was difficult in histochemically stained sections [6]. There has been great interest in methods that selectively identify specific cell populations without degrading mRNA during tissue processing. The recent introduction of immuno-LCM extended the ability to isolate cells based upon their expression of function-related proteins or specific immunophenotype [3]. Frozen sections are rapidly immunostained and then subjected to LCM, RNA extraction, and PCR. The total aqueous time is shortened to 8 to 10 minutes: 4 to 5 minutes of antibody exposure and 4

to 5 minutes of color development. Although immunohistochemistry staining protocols were optimized to reduce the time of aqueous incubation and hence lessen mRNA degradation, 99% of the mRNA for β-actin was lost [5, 6]. RNA loss occurred even in the absence of antibody, suggesting that endogenous RNases were not inhibited sufficiently during the aqueous phase incubations.

Immunofluorescence methods offer several advantages over immunohistochemical methods. First, immunofluorescence methods are more sensitive and thus can detect lower concentrations of antigens. Second, immunofluorescence methods do not require additional enzymatic reactions for visualization. We hypothesized that immunofluorescent labeling could be preformed more rapidly, thus allowing less time for RNA degradation. Therefore, we developed a rapid immunofluorescence LCM (IF-LCM) procedure that allows targeted analysis of gene expression in specific cells from frozen sections. Studies were performed in kidney tissue, since the kidney is an anatomically complex organ with exceptional cellular heterogeneity.

METHODS

Modifications to LCM microscope for immunofluorescence

A Pixel II LCM microscope (Arcturus Engineering Inc., Mountain View, CA, USA) was modified for fluorescence excitation, low light level image detection, and transmitted light image detection. The fluorescent excitation light was provided by a mercury light source (BH2-RFL-T3; Olympus, Tokyo, Japan) and a FITC filter cube (OM-XF 100; Opelco, Sterling, VA, USA). The low light level immunofluorescence image was captured by an image intensifier (KS-1380; Opelco) coupled to a video camera (CCD 72; Dage MTI, Michigan City, IN, USA; or Model 1322-1000; Coho, San Diego, CA, USA) on the side port of the microscope. This intensifier adds 10^4 to 10^5 gain to the detection system [9]. Two infrared heat blocking filters (51962; Oriel, Stratford, CT, USA) were placed between the intensifier and the microscope to decrease the intensity of the tracking laser and prevent blooming of the laser spot. Standard transmitted light color images were recorded with a color video camera attached to a trioocular microscope head (Olympus).

Animals

Female Balb/c mice, six weeks old, were purchased from the National Institutes of Health. All animals had free access to water and food. All animal procedures were approved by the National Institute of Diabetes and Digestive and Kidney Diseases Animal Care and Use Committee. Mice were anesthetized with 1000 mg/kg ketamine, 20 mg/kg xylazine, and 10 mg/kg acepromazine injected intramuscularly Both kidneys were harvested and immediately frozen with OCT Compound (62550; Sakura-Finetek, Torrance, CA, USA).

Qualitative changes in tissue RNA level

Acridine orange stains RNA with an orange color [10, 11]. Sections of mouse kidney were fixed with ice-cold acetone for two minutes and were incubated in diethel pyrocarbonate (DEPC)-treated water at room temperature for 1, 3, 5, or 10 minutes. The sections were stained for 30 seconds with 0.02% acridine orange (2 mg in 10 mL 0.067 mol/L phosphate buffer, pH 6.0) at room temperature and were then washed with phosphate-buffered saline (PBS)/DEPC-treated water (pH 6.0) for 30 seconds. The sections were examined immediately using an ultraviolet filter cube and the image captured with a color video camera.

Immunofluorescence-LCM

The primary antibody was monoclonal anti–Tamm-Horsfall protein (THP) antibody (CL1032A; Accurate Chemical & Scientific Corp., Westbury, NY, USA); the secondary antibody was goat anti-mouse IgG antibody (A-11029; Molecular Probes, Eugene, OR, USA). High concentrations of primary antibody (1:20 dilution; 20-fold greater than routine immunohistochemistry), secondary antibody (1:7 dilution), and RNAse inhibitor (400 U/mL; N251A; Promega, Madison, WI, USA) were premixed for 10 minutes at room temperature. Freshly frozen tissue sections were fixed for two minutes with cold acetone and then washed twice five seconds each with DEPC-treated PBS, pH 7.6. The sections were incubated with premixed primary and secondary antibody for one minute. The sections were washed rapidly two times for five second each with PBS/DEPC at room temperature, dehydrated, and air dried. Slides were viewed with a FITC filter cube and low light level video microscopy, as described previously in this article. Specific portions of the histologic section were affixed to transfer film (CapSure TF-100; Arcturus Engineering Inc.) by brief laser pulses as previously described [3]. As a control, we incubated sections with a different primary antibody (M085; Dako, Carpinteria, CA, USA) or with only the secondary antibody.

Hematoxylin and eosin staining

The sections were fixed with 70% ethanol for two minutes and then washed with DEPC-treated water for five seconds. Sections were stained rapidly with hematoxylin stain (CS 401-1D; Fisher Scientific, Pittsburgh, PA, USA) for 30 seconds, washed with DEPC-treated water for 10 seconds, dehydrated with an ethanol gradient, and counterstained with alcoholic Eosin Y solution (HT110-1-16; Sigma Chemical Co., St. Louis, MO, USA) for 30 seconds. Sections were washed three times with 100% ethanol and were then washed two times with xylenes and air dried.

Table 1. Sequences of oligonucleotide primers

Gene	Gene Bank identifier	Oligonucleotide sequence	Location (bp)	Product
Mouse MDH	(M29462)			
5′ sense		GGTCATTGTTGTGGGAAACC	(456–475)	431
3′ sense		TCGACACGAACTCTCCCTCT	(867–886)	
Mouse AQP2	(AF020519)			
5′ sense		ATGTGGGAACTCCGGTCCATA	(1–21)	477
3′ sense		GCTACCCAGGTTGTCACTGC	(458–477)	
Mouse THP	(L33406)			
5′ sense		TCAGCCTGAAGACCTCCCTA	(1430–1449)	230
3′ sense		TGTGGCATAGCAGTTGGTCA	(1640–1659)	
Mouse BAT	(D88533)			
5′ sense		ACGTCTTCCTCGTGGTTCTG	(1814–1833)	315
3′ sense		GGCATCTCTTAGGGAGCTT	(2110–2128)	

Genes are identified by GeneBank abbreviations. Abbreviations are: bp, base pair; MDH, malate dehydrogenate; AQP2, aquaporin-2; THP, Tamm-Horsfall protein; BAT, dibasic and neutral amino acid transporter.

RNA extraction and reverse transcription

Total RNA was extracted from samples attached to the LCM transfer film using GTC/phenol-chloroform as previously described [6]. The sample film was incubated with 200 μL of 4 mol/L guanidine thiocyanate, 25 mmol/L Na_3 citrate, 0.5% sarcosyl, and 0.72% β-mercaptoethanol for 10 minutes at room temperature. After centrifuging samples, the GTC solution was removed to a new 500 μL test tube, and 200 μL of the phenol-chloroform were added. Samples were vortexed and centrifuged 30 minutes at 4°C. The aqueous layer was transferred to new 500 μL test tube and extracted again with phenol-chloroform. The samples were washed with chloroform and precipitated with isopropanol. Samples were frozen for one hour at −80°C and centrifuged 40 minutes. Samples were washed with 70% ethanol and then 100% ethanol and then were air dried. Sample RNA was resuspended with 10.5 μL of resuspension solution [7.1 mmol/L dithiothreitol (DTT), 1.7 U/μL recombinant RNase inhibitor in DEPC-treated water]. Seven microliters of the RNA solution were denatured at 60°C for 10 minutes and mixed with 12 μL of RT master mix (final concentration 3 mmol/L Mg^{2+}, 1 mmol/L dNTP, 5 mmol/L DTT, 1.35 U/μL RNAsin, 5 μmol/L oligo dT15) with 1 μL (200 units) of MMLV-RT (Promega). RT reaction was performed at 42°C for 60 minutes. The remainder of the RNA solution was processed without reverse transcriptase (−RT).

Polymerase chain reaction

Reverse transcriptase products were used as template for PCR as previously described [6]. We chose primer sets that hybridized to different exons and produced a single correctly sized band (Table 1). Since the BAT primer set was in the same exon, a portion of the RNA sample was treated with DNAse I for one hour. PCR reactions contained 1 mmol/L primers, 1 to 1.5 mmol/L Mg^{2+}, 200 μmol/L deoxynucleotide triphosphates, reaction buffer, and 1.5 units Taq DNA polymerase (Promega) in a final volume of 25 μL. PCR was performed using 35 cycles of 94°C for 45 seconds, 55°C for 45 seconds, and 72°C for 1.25 minutes. Reaction products were size fractionated by gel electrophoresis and ethidium bromide staining.

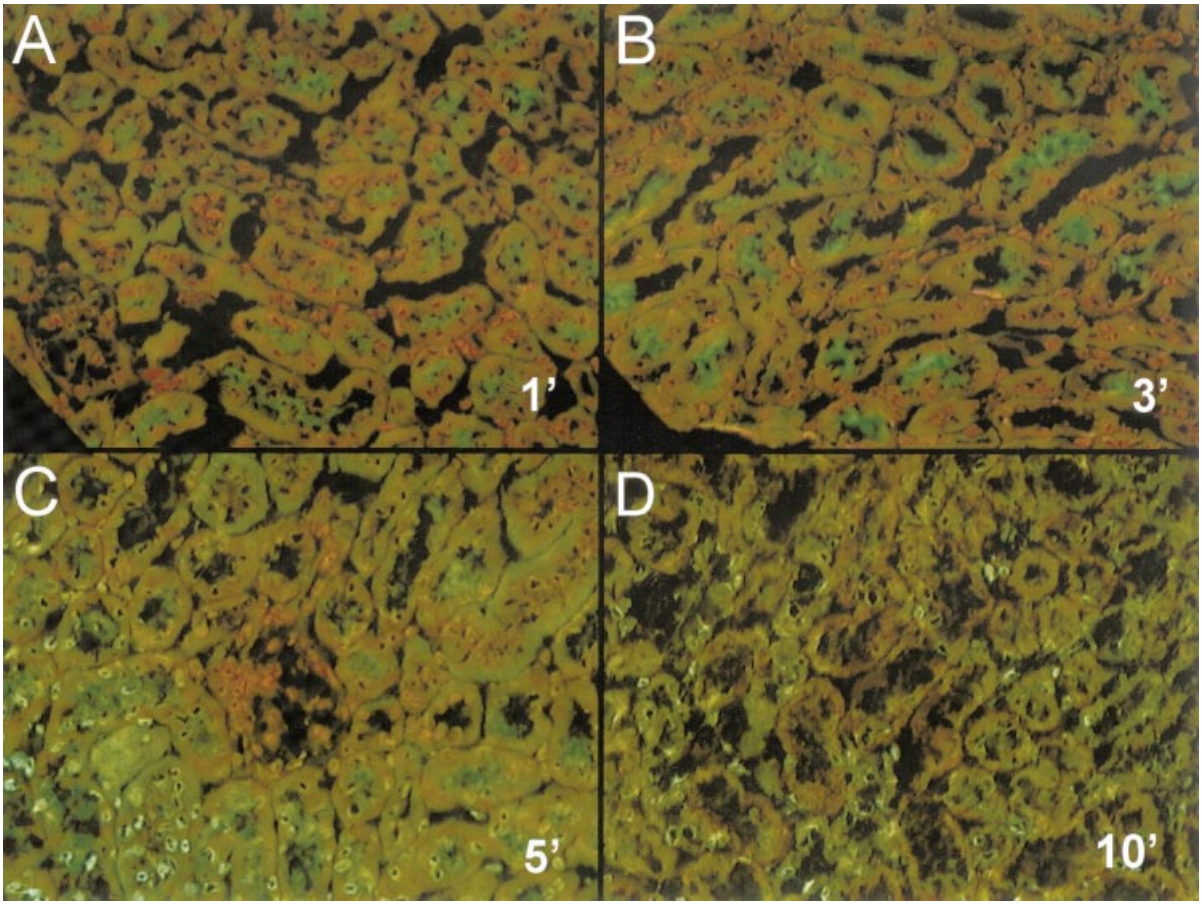
RESULTS

Temporal changes in tissue RNA and mRNA

We found previously that 8 to 10 minutes of incubation with aqueous solutions destroyed 99% of mRNA for β action [6]. Therefore, we examined the disappearance of RNA and mRNA. Tissue RNA was measured using acridine orange, which stains RNA orange [10, 11]. Sections of mouse kidney were incubated in DEPC-treated water for 1, 3, 5, or 10 minutes and then stained with acridine orange. Figure 1 shows that tissue RNA decreases rap-

Fig. 1. Time course of RNA disappearance by acridine orange. Sections of mouse kidney were fixed in ice-cold acetone, incubated in diethel pyrocarbonate (DEPC)-treated water for 1, 3, 5, or 10 minutes at room temperature, and then rapidly stained with 0.02% acridine orange. After a brief washing, the sections were examined using an ultraviolet filter cube, and the image was captured with a Coho video camera.

Fig. 3. Laser capture microdissection capture of immunofluorescently stained mouse kidney. A 2 μm section of freshly frozen mouse kidney tissue was fixed in 100% acetone and rapidly immunostained with anti-Tamm-Horsfall protein (anti-THP; **Methods** section). (*A* and *B*) The immunofluorescent and transmitted light image before transfer, respectively. (*C*) The fluorescent image of the tissue section after dissection. (*D*) A fluorescent image of the thick ascending limb (TAL) recovered on the transfer film. The arrow indicates the transferred area.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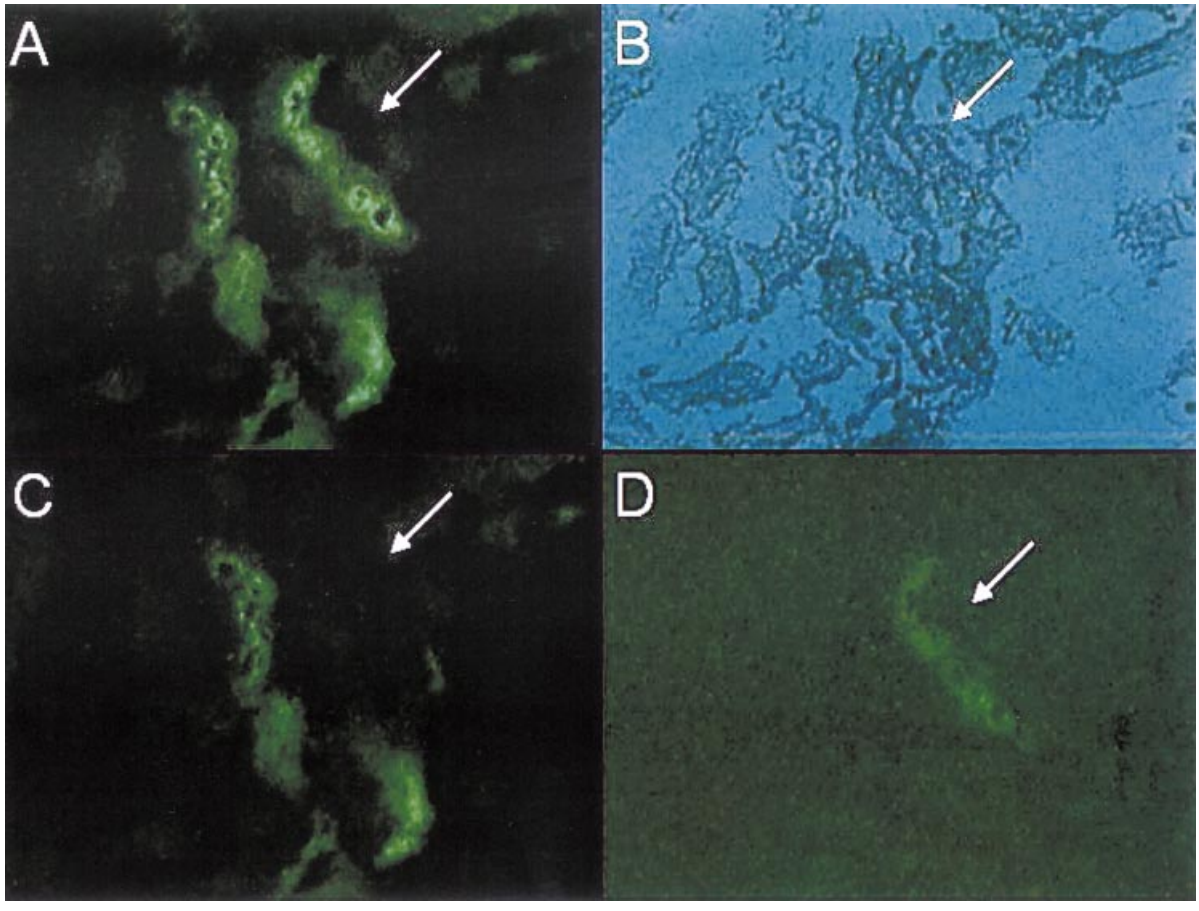
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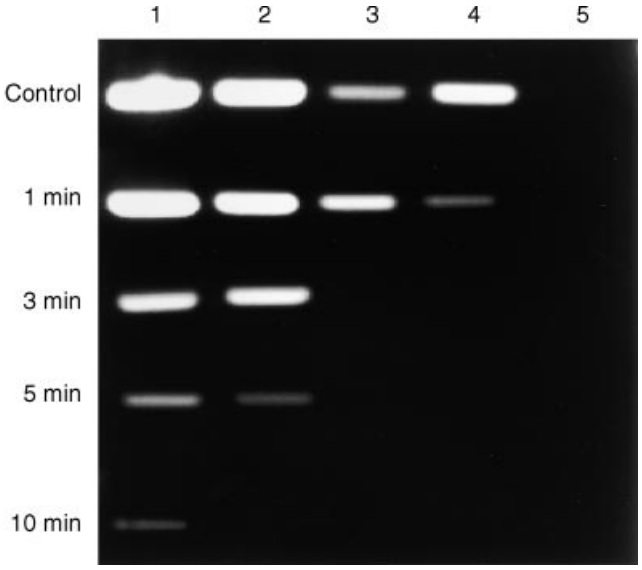

Fig. 2. Time course of RNA disappearance of reverse transcription-polymerase chain reaction (RT-PCR). Cryosections (2 μm) of a mouse kidney were exposed to 0 (control), 1, 3, 5, or 10 minutes of DEPC-treated water at room temperature, dehydrated, and air dried, and then manually scraped with a clean razor blade as described previously [6]. The RNA was extracted, and RT-PCR was performed for malate dehydrogenate (MDH). Columns are successive 1:10 dilutions of cDNA obtained from the entire section. MDH mRNA was not detected in the one-minute sample processed without RT (data not shown).

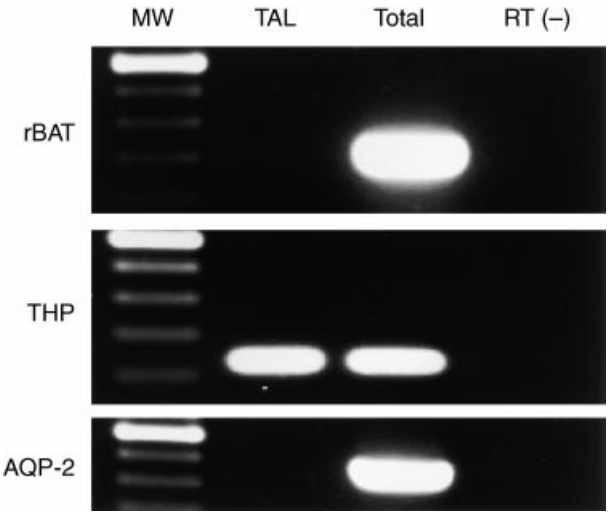

Fig. 4. Specificity of IF-LCM capture. RT-PCR analysis of three genes in TAL sample isolated by LCM. Mouse outer medullary TALs were labeled with THP as in Figure 3. All the TALs on a single kidney cross-section were microdissected using approximately 700 7.5 μm diameter laser spots. Each RT-PCR reaction used 7% of the RNA obtained from all of the TALs identified on a single kidney cross-section. Table 1 shows the details of PCR primers and reaction conditions. Abbreviations are: rBAT, basic amino acid transporter; THP, Tamm-Horsfall protein; AQP-2, aquaporin-2. Positive control (total), pooled sample of RNA harvested from scraped section from same block. Negative control [RT(−)], total RNA from microdissected TAL analyzed without reverse transcriptase.

idly under these conditions. We then determined the time course of tissue mRNA recovery using semiquantitative RT/PCR for malate dehydrogenate (Fig. 2). Several cryosections were exposed to 0 (control), 1, 3, 5, or 10 minutes of DEPC-treated water, were dehydrated and air dried, and finally were manually scraped with a clean razor blade. RNA was extracted, and RT-PCR was performed for malate dehydrogenate (MDH). The semiquantitative RT-PCR results demonstrate that one-minute exposure to aqueous solutions (without RNAse) had the best preservation of tissue mRNA. The different time courses of tissue total RNA (by acridine orange) and tissue mRNA (by RT-PCR) suggest that RNAse-induced cutting of mRNA is faster than diffusion of RNA from the tissue. Both studies suggest that the immunofluorescent staining must be performed very rapidly. Therefore, subsequent studies were performed using a one-minute antibody binding step followed by rapid washing.

Detection and selective transfer of thick ascending limb

Figure 3 shows the detection, transfer, and recovery of outer medullary thick ascending limbs (TALs) by LCM. Normal mouse kidney sections were fixed in acetone and rapidly immunostained with one-minute exposure to premixed primary and secondary antibody. We chose anti-THP because it specifically stains TALs [12]. The TALs were difficult if not impossible to detect in unstained frozen sections examined by transmitted light (Fig. 3B), but were easily seen by immunofluorescence after labeling with THP (Fig. 3A). The labeled portion of the tubule was transferred by overlapping 7.5 μm laser spots (Fig. 3 C, D). There was no apparent contamination by surrounding tissue at the level of light microscopy (Fig. 3D). In control experiments (data not shown), we did not detect staining of the TAL with either omission of the primary antibody or a different secondary antibody (monoclonal anti-human α-smooth muscle actin antibody). The latter antibody did stain smooth muscle cells in the media of renal blood vessels.

To evaluate whether IF-LCM can selectively isolate TALs, we microdissected many TAL (using approximately 700 7.5 μm diameter laser spots) and tested the sample with a panel of genes that are expressed in a segment specific fashion: Basic amino transporter (BAT) expressed proximal convoluted and straight tubules [13]. THP expressed only in TAL [12], and aquaporin-2 (AQP-2) expressed only in the collecting duct system [14]. We detected THP as expected (Fig. 4), but could not detect BAT or AQP-2, suggesting that the microdissected cells were not contaminated by surrounding proximal tubules (BAT) or collecting ducts (AQP-2). This indicates that the cells were TAL cells, thus confirming the specificity of the cell identification and microdissection.

Recovery of mRNA during IF-LCM

We compared the recovery of mRNA during conventional hematoxylin and eosin (HE) staining LCM and rapid IF-LCM. Alternative sections of the same block were stained with rapid HE or rapid IF protocols and were scraped, and RT-PCR was performed for MDH (Fig. 5). Semiquantitative RT-PCR confirmed that the two procedures had similar excellent preservation of tissue RNA levels.

DISCUSSION

Preservation of mRNA during tissue fixation, sectioning, and staining has been easy to achieve because these steps can be performed in nonaqueous solutions. However, it has not been known how to identify cells antigenically while preserving most of the mRNA. Previous methods have relied on immunohistochemistry, which incorporates lengthy enzymatic reactions that must be carried out in aqueous solutions [3]. We found that exposure to aqueous solutions for more than a few minutes was sufficient to cause loss of tissue RNA and mRNA (Figs. 1 and 2) [6]. Indeed, acridine orange staining may be useful as a screening test for RNA preservation. Sections or blocks lacking orange color should not be used for LCM; however, sections with orange color could still have poor mRNA preservation since tissue mRNA decays more rapidly than tissue RNA. The IF-LCM method reported here required the development of a protocol for rapid immunofluorescence labeling of desired cells, and optical modifications for immunofluorescence image detection by low light level video microscopy and introduction of heat filters to prevent the tracking laser light from blooming on the detector. We found that IF-LCM could selectively identify and isolate a defined portion of the nephron that is difficult to identify by light microscopy in unstained tissue. Using these modifications, we were able to achieve excellent delineation of the tagged cells and excellent recovery of mRNA from the tissue sections. These modifications are discussed later in this article.

Rapid immunofluorescence protocol

We [6] and others [15] have optimized the fixation and tissue processing step prior to LCM and found that acceptable tissue morphology and optimal mRNA recovery was obtained with frozen tissue that was fixed with non–cross-linking fixatives such as ethanol, acetone, or methanol and lightly stained with HE. Therefore, the present study was performed on frozen tissue fixed with acetone. Immunologic labeling of cells usually includes two 30- to 60-minute incubation steps that allow the primary and secondary antibodies sufficient time to bind the intended target. Immuno-LCM as described by Fend et al shortened the primary and secondary antibody binding reactions to 1.5 to 2.0 minutes each [3]. However, the eight minutes required for rapid immunofluorescence resulted in 99% loss of tissue mRNA levels (Fig. 2) [6]. Approximately half the time required for immunohistochemical labeling is spent in the color development step. Therefore, we switched to an immunofluorescent detection scheme and shortened the aqueous exposure time by four minutes. An ALEXA-conjugated secondary antibody was used because the fluorescent signal survives the ethanol and xylene dehydration. We then optimized all of the remaining aqueous steps. We combined the primary and secondary antibody labeling reaction steps, which reduced the aqueous exposure to one minute without jeopardizing the ability to detect the fluorescent label in tissue sections by low light level video microscopy. The washing steps were also shortened to rapid in and out dipping of the slides in the wash solutions. With these modifications, we could complete the aqueous phase steps in 1.3 minutes, yet still obtain both excellent immunofluorescent labeling (Fig. 3) and mRNA recovery (Fig. 5).

We have also used this method successfully with ALEXA-conjugated primary polyclonal antibodies (data not shown). We found that monoclonal antibodies and antibodies that gave strong signals with conventional immunofluorescence protocols worked best, with the least background (data not shown). Antibodies that require long incubation times may not be appropriate for IF-LCM. The sensitivity of the low light level video detection may allow use of even weak antibodies.

Optical path modifications

The light generated by conventional immunofluorescent staining methods is too faint to be detected with standard color video camera. The rapid immunofluorescence staining method produces even dimmer images. Therefore, we placed a KS-1380 image intensifier between the LCM microscope and the video camera (either a conventional color camera or sensitive black and white camera). The KS-1380 image intensifier has a maximum gain of 10^4 to 10^5; we typically operated the intensifier at about one third of maximum gain. The low light level video system could easily detect the fluorescent light generated by both conventional and rapidly stained immunofluorescent samples. Although fainter signals could potentially be detected, detection of immunofluorescently labeled cells appeared to be limited more by nonspecific labeling of surrounding tissue than by the weakness of the specific signal. Images with marginal signal to background signals can be amplified by adjusting the dark current of the video camera so that the background nonspecific binding becomes black or by using false coloring schemes to bring out the specific signal.

The KS-1380 image intensifier is very sensitive to the laser light, and we found intense blooming (enlarging) of the laser image on the intensifier. Therefore, we added several heat filters between the microscope and the im-

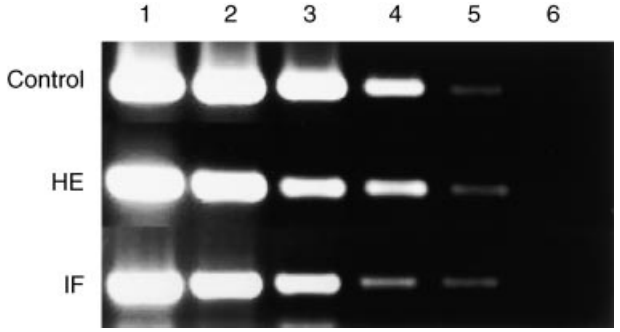

Fig. 5. mRNA recovery following immunofluorescent (IF) and hematoxylin and eosin (H&E) staining. Serial 2 μm cryosections of mouse kidney were fixed with acetone and dehydrated (without exposure to aqueous solutions, control), rapidly immunofluorescently labeled with THP, or stained with HE. Slides were scraped. RNA was extracted, and RT-PCR was performed for MDH mRNA as in Figure 2. Each RT-PCR reaction tube used 7% of the RNA obtained from a single 2 μm cryosection.

age intensifier. This protected the image intenser from laser light induced damage and also allowed us to focus the laser using the same imaging system we used to identify the fluorescently tagged cells.

Uses of IF-LCM

The ability to immunoselect a cell or cell population prior to microdissection extends our ability to isolate and investigate the function of pure populations of cells. It may be difficult to identify specific populations of cells from frozen sections of anatomically complex organs or tumors even with knowledge of detailed anatomy. We found that immunofluorescent labeled TAL tubules could be easily identified, thus dramatically speeding up the microdissection. LCM has been used to study cellular genomic DNA, mRNA, and proteins. At the genomic DNA level, it has been used to detect changes in tumor heterozygosity and preneoplastic changes in tissues surrounding tumors [1]. At the RNA level, LCM can be used to measure mRNA abundance of individual genes, generate expression libraries, perform subtractive hybridization cloning, or screen high density cDNA arrays [2, 7, 8, 16, 17]. Recent studies have now extended LCM to measuring changes in cellular proteins. Tissue obtained by LCM can be analyzed with sensitive immunoassays, two-dimensional gel electrophoresis, or protein chips [18–21]. For example, LCM has detected heterogeneity of the prostate specific antigen content of prostate carcinoma cells in an individual patient [21]. Thus, LCM can be used to determine the functional state of a cell at the genomic, RNA, or protein level. IF-LCM extends the capability of any of these methods by allowing cells to be selected and microdissected on the basis of expressed functional proteins or previously characterized immunologic determinates.

Laser capture microdissection has been used to obtain specific populations of cells from fibrotic or injured tissue [6]. The IF-LCM technique can be used in injured tissues if the cells remain sufficiently differentiated to keep their specific markers. Tamm-Horsfall mRNA is known to decrease following renal ischemia [22]; we found that detection of ischemic TALs with this antibody was difficult. However, the Na-K-2 Cl cotransporter remains high following ischemia [23] and thus might be useful for isolating ischemic TALs. Thus, judicious choice of phenotypic markers is critical to the success of IF-LCM.

This method may also be useful to procure green fluorescence protein (GFP)-labeled cells from transgenic animals, transected organ cultures, or GFP-transected reporter cells that are injected into animals. Chemical or induced fluorescence without antibodies should be detectable with this high sensitivity detection system.

Conclusions

We conclude that specific cells can be identified and microdissected by IF-LCM. The rapid immunofluorescent reactions can be completed before there is significant loss of mRNA. Low light level detection allows the weakly stained sections to be visualized. This method extends the ability to isolate pure populations of immunotypically defined cells from a sea of similarly appearing cells and process for further analysis to determine the functional state of the cells.

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