Laser Capture Microdissection of Cells from Plant Tissues[1]

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Laser capture microdissection (LCM) is a technique by which individual cells can be harvested from tissue sections while they are viewed under the microscope, by tacking selected cells to an adhesive film with a laser beam. Harvested cells can provide DNA, RNA, and protein for the profiling of genomic characteristics, gene expression, and protein spectra from individual cell types. We have optimized LCM for a variety of plant tissues and species, permitting the harvesting of cells from paraffin sections that maintain histological detail. We show that RNA can be extracted from LCM-harvested plant cells in amount and quality that are sufficient for the comparison of RNAs among individual cell types. The linear amplification of LCM-captured RNA should permit the expression profiling of plant cell types.

Methods such as immunolocalization, in situ hybridization, and reporter gene visualization have permitted the cell-specific analysis of the expression of individual genes and of the accumulation of individual proteins. New methods promise to provide such cellular information on a genome- and proteome-wide scale. However, the specificity of information derived from RNA and protein expression profiling is limited to the specificity of the biological starting material. It has been challenging to obtain cell preparations of single types, developmental stages, and/or unique locations from plants, and thus most profiling has only been possible with whole-tissue resolution. Schemes for isolating specific cells thus far rely on extensive manipulation (e.g. tissue digestion and cell sorting) and in some cases rely on the prior identification of cell-specific markers. Laser capture microdissection (LCM) provides a rapid means of isolating pure cellular preparations directly from heterogeneous tissues, based on conventional histological identification (Simone et al., 1998). Specific markers can assist with the identification of the desired cells, before or after isolation, but they are not a requirement for LCM itself.

In the LCM version developed at the National Institues of Health (Emmert-Buck et al., 1996) and commercially available as the Pix-Cell system (Arcturus Engineering, Mountain View, CA; http://www.arctur.com), a HeNe laser beam is used to tack selected cells to a thermoplastic film suspended above a tissue slice while it is viewed on an inverted microscope. The film is attached to an optically clear microfuge tube cap, and cells harvested onto the cap can be subjected to high-efficiency procedures for the isolation and analysis of DNA, RNA, and protein. Several similar methodologies employ laser pressure catapulting, laser ablation excision, electrostatic harvest, and other variations (for review, see Roberts, 2002), all with the aim of removing visually identified cells from tissue slices. A variety of proof-of-concept and analytical studies have demonstrated that the DNA, RNA, and protein obtained from LCM-harvested cells can be used for microarray-based RNA expression profiling, proteomic protein profiling, and genomic mutational analysis (Banks et al., 1999; Jin et al., 1999; Luo et al., 1999; Ohyama et al., 2000; Simone et al., 2000; Wong et al., 2000). In addition to numerous studies with frozen or freshly fixed and sectioned samples, the LCM method has made possible the recovery of cell-specific materials from specimens archived in paraffin blocks, although with lower efficiency than from cryosectioned tissue (Goldsworthy et al., 1999). Most studies using LCM have thus far used animal tissues as subjects, and the reported methods for the fixation, sectioning, visualization, and extraction of macromolecules in LCM experiments have been based on protocols optimized for animal cells (Goldsworthy et al., 1999).

Tissue preparation for any localization study requires a balance between the preservation of histological detail and preservation of access to the probed features in a state sufficiently native for detection. For LCM, the aim is to preserve enough visual detail to identify specific cells for harvest, yet allow the maximum subsequent recovery of RNA, DNA, or protein from the harvested cells. Histological methods for plant tissues differ significantly from those commonly used for animal tissues, as a result of the

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differing structures and compositions of plant and animal cells. A difference relevant to LCM is the presence of vacuoles and cell walls in plants. Some methods proven to be useful for LCM of animal tissues, such as cryosectioning, are undesirable for many plant tissues because of the difficulty of stabilizing vacuolated cells and the loss of tissue integrity caused by freezing and thawing. This compromises the ability to identify cells on the basis of their histological appearance. Recently, a variation of laser pressure catapulting was used to harvest rice (*Oryza sativa*) phloem tissue from cryosections, although the general applicability and cellular resolution of this method was not described (Asano et al., 2002). The highly vacuolated nature of many mature plant cell types also means that the nucleus and cytoplasm may appear as a small target relative to the total cross-sectional area of the cell.

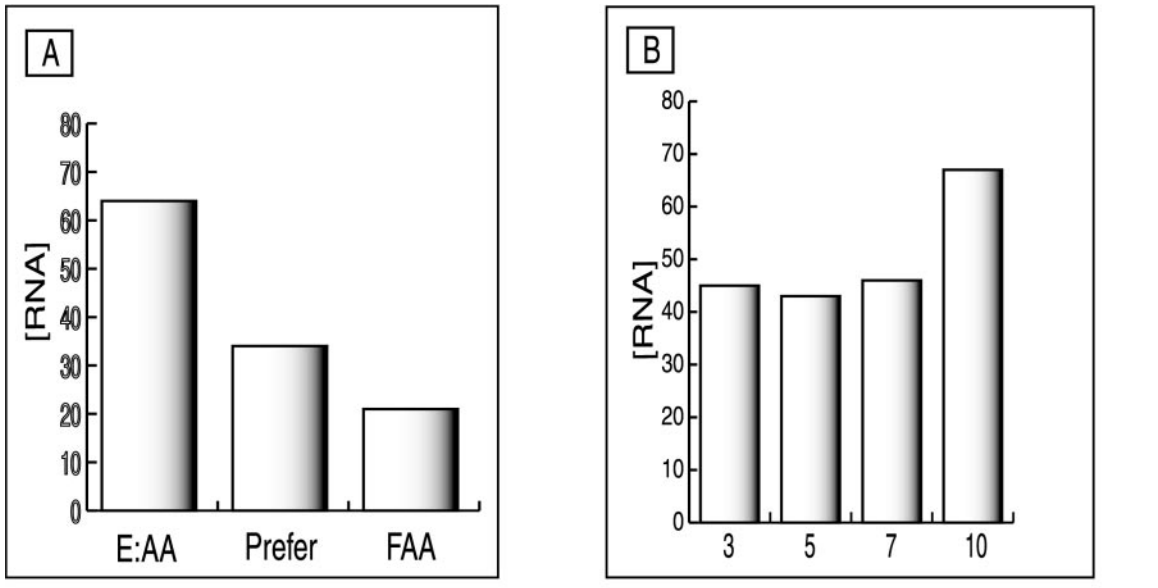
Here, we provide a scheme for adapting LCM to plant tissues, with the cellular resolution and stability afforded by paraffin-embedded tissue sections. We demonstrate that the technique permits the isolation of cell-specific RNA from complex tissues of various organ systems from several plant species. Such plant cell-specific RNA is suitable for amplification into probes for expression profiling, as demonstrated by recent profiling studies that used amplified RNA isolated from individual plant cells by micropipetting (Brandt et al., 2002).

RESULTS

Preparation of Plant Tissues for LCM

Fixation

Fixation is needed to stabilize the cell contents and to preserve histological integrity and detail during tissue sectioning and cell harvest. Unfortunately, this competes with the need to extract cell contents after harvest. Precipitative fixatives were found to be preferable for the recovery of RNA from animal cells captured from cryosections by LCM (Goldsworthy et al., 1999). Consequently, we compared the effects of plant tissue precipitative and cross-linking fixation methods on the recovery of RNA from several different cell and tissue types, from several species. All of the methods tested provide adequate preservation of tissue structure for the identification of individual cell types for single-cell harvests. However, we recovered two to three times more RNA from cells harvested from tissues prepared with the precipitative fixative ethanol-acetic acid than from those treated with the cross-linking fixatives formaldehyde-acetic acid-ethanol (FAA) and Prefer (proprietary formula; Fig. 1A). This suggests that a significant portion of cellular RNA is cross-linked or otherwise obstructed from extraction by FAA treatment. The fixation condition provided in "Materials and Methods" should be considered a starting point for the optimization of



	E:AA	Prefer	FAA
ng RNA	64 ± 3	34 ± 5	21 ± 11

thickness	3 μm	5 μm	7 μm	10 μm
ng RNA	45 ± 0.2	43 ± 5	46 ± 1	67 ± 20

Figure 1. A, Comparison of RNA recovery using different fixatives: ethanol-acetic acid (E:AA), Prefer, and FAA. B, Comparison of RNA recovery from sections of different thickness as indicated below the graph. The means and SDs of three independent experiments are shown in the table below the graphs.

the balance between RNA recovery and tissue preservation for a new tissue source.

Embedding and Sectioning

For reasons cited above, we paraffin-embedded tissues to prepare sections for LCM. In addition to providing superior visual aid to cell harvest, this approach has the advantage that embedded tissues are stable over time and need not be harvested immediately. The block serves as an archive that can be resampled or from which serial sections can be stained or treated with antibodies or other reporters to identify specific cells for harvest. Such archiving and repeated sampling is generally impractical for frozen samples.

We tested whether the thickness of the tissue section relative to the average cell diameter (in depth) had a significant effect on the success of harvest of cells from various tissues. The cell walls might present a significant barrier to access and/or release of individual cells from the top surface of the section, if the section is greater or less than the average cell thickness. We compared sections of radish (*Raphanus sativus*) cortical parenchyma varying in thickness from 3 to 10 μm (Fig. 1B) and found that section thickness in this range had little effect on the recovery of RNA from harvested cells. We standardized on a section thickness of 10 μm for mature tissues and 6 μm for developing tissues with smaller cells.

Paraffin-embedded materials were stable sources of cells and RNA. Unsectioned blocks of paraffin-embedded tissue remained suitable for sectioning, LCM, and RNA isolation for at least several weeks if stored at 4°C in the presence of desiccant. Slides with fixed, and sectioned specimens were suitable for subsequent LCM for at least 2 weeks if stored before deparaffinization in the presence of desiccant at 4°C. Samples should be deparaffinized immediately before the LCM procedure. After LCM, the adhesive caps with their captured cell samples can be stored at −80°C in the initial buffer for subsequent RNA isolation for at least several months before the extraction is completed.

Cells Can Be Harvested by LCM from Many Organ Systems and Species

We used LCM to harvest cells from paraffin sections of a variety of organ systems and species, adjusting beam size, power, and duration to adhere one cell per laser burst to the film. We were successful in harvesting every cell type and tissue tested, based on visual recovery, although not all were tested by subsequent RNA extraction and analysis (Fig. 2). The stability and detail provided by paraffin sections makes possible considerable precision in cell harvest. Multiple samplings can be made of a single tissue section, to remove different cell types to separate harvesting caps, or to remove potentially contaminating cells from the vicinity of a few desired cells. Complex structures such as shoot apices and organ primordia can be microdissected into cells from individual constituent cell layers.

Plasmolysis

The vacuole occupies a significant volume in many plant cell types, in some mature cells limiting the cytoplasm and nucleus to a thin layer along the plasma membrane. In cases in which it is important to avoid contamination with adjacent cells, it is possible to shrink the protoplast away from the shared wall by means of plasmolysis. We tested whether the disruption of the vacuolar volume by 4-h treatment in 1 M mannitol had an effect on the harvest of mature bundle sheath (BS) cells from maize (*Zea mays*) leaves and the subsequent recovery of RNA. This plasmolysis treatment concentrates the cytoplasm and organelles into a collapsed mass, facilitating its harvest by LCM (Fig. 3). Although the disruption of the vacuole might be expected to release hydrolytic enzymes, we were able to isolate intact RNA from cells plasmolysed before harvest.

Extraction of RNA from Harvested Cells

There are numerous public-domain and proprietary commercial methods for the isolation of RNA from small samples. We compared four representative high-efficiency RNA extraction methods with regard to RNA yield from plant cells harvested onto Pix-Cell film microfuge caps (Arcturus Engineering; Fig. 4). These were an optimized phenol extraction method (TRIzol, Invitrogen, Carlsbad, CA) and three resin spin-column systems (Nanoprep and Miniprep, Stratagene; PicoPure, Arcturus Engineering). RNA yield from Pix-Cell caps was highly dependent on the extraction method used, with the PicoPure system providing the highest yield. Results for the two Stratagene kits were comparable, and only data from Miniprep is shown. For all four methods, the amount of RNA recovered from individual caps was directly proportional to the number of cells harvested, down to 25 to 50 cells (Fig. 4A). The four RNA extraction methods also varied in the amount of DNA that contaminated the extracted RNA preparations, as determined by subsequent DNase treatment (Fig. 4C). The TRIzol method yielded DNA-free RNA, whereas DNA remained in extractions using the PicoPure method (Fig. 4D). However, the amount of RNA extracted by the PicoPure method was consistently greater than with other methods, when measured after DNase treatment. It should be noted that the PixCell film is dissolved by the TRIzol extraction but is resistant to the other methods. For some extraction procedures, it is possible to extract several caps se-

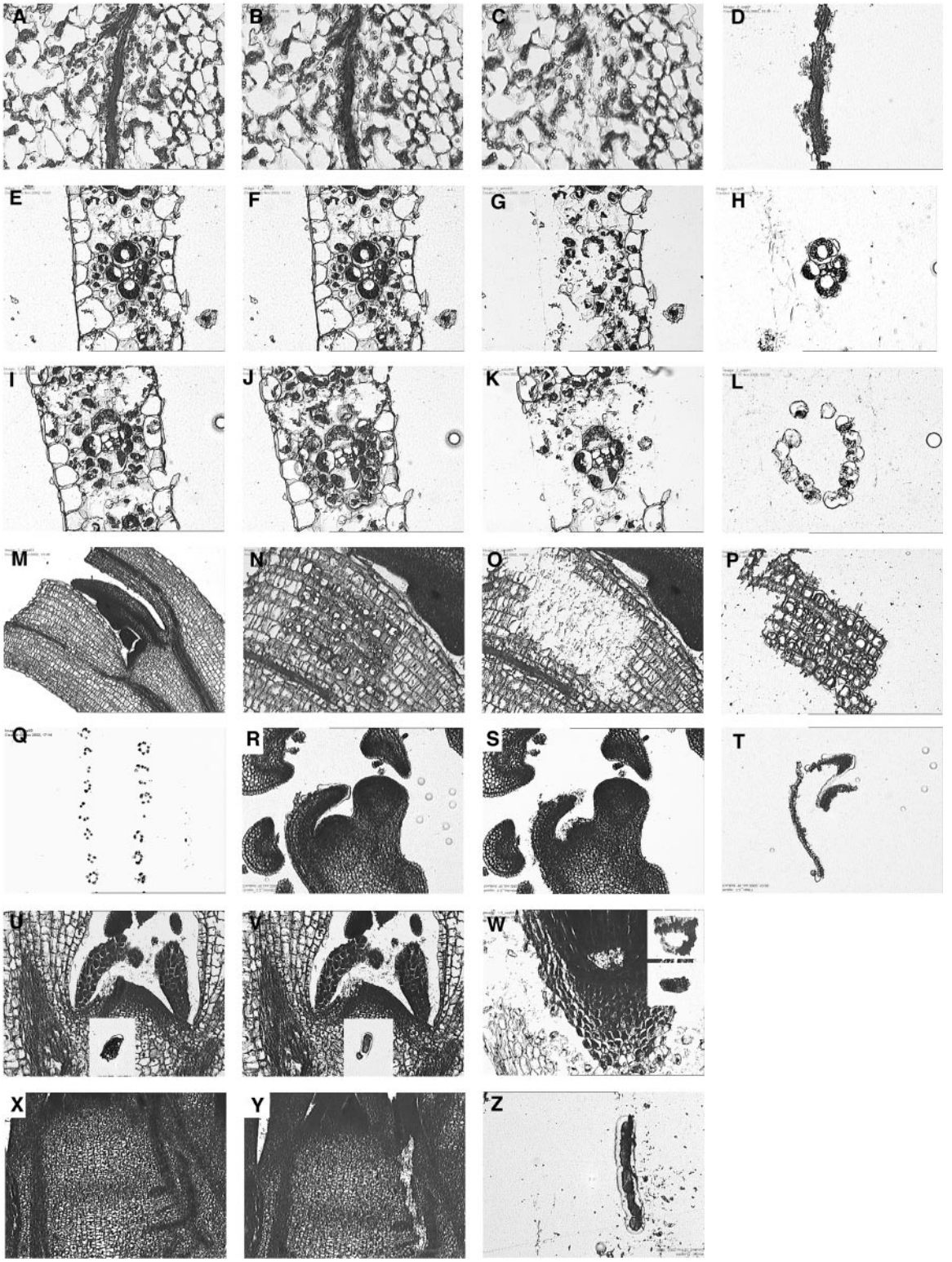


Figure 2. Laser capture of various cell and tissue types. A through D, Arabidopsis cotyledon procambium capture: A, before laser; B, after laser pulses; C, tissue remaining after capture; and D, procambium captured on cap. E through H, Capture of maize BS cells: E, before laser; F, after laser pulses; G, tissue remaining after capture; and H, BS cells captured on cap. I through L, Capture of maize mesophyll cells: I, before laser; J, after laser pulses; K, tissue remaining after capture; and L, mesophyll cells captured on cap. Laser beam size is evident in I, J, and L. M through P, Capture of radish seedling petiole cells: M, before laser; N, after laser pulses; O, tissue remaining after capture; and P, parenchyma cells captured on cap. Q,

(*Legend continues on facing page.*)

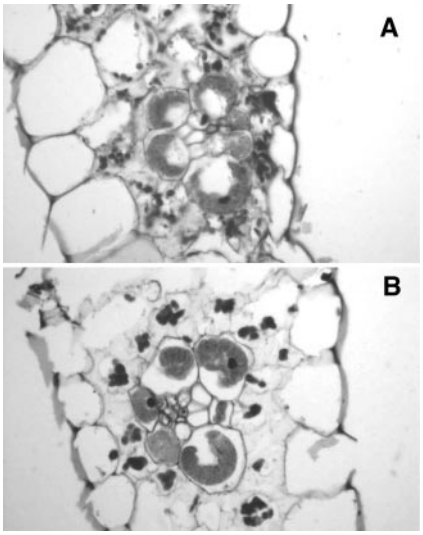


Figure 3. Effect of plasmolysis on maize leaf cells. A, Control leaf incubated in water for 4 h before fixation. B, Leaf incubated in 1.0 M mannitol for 4 h before fixation (toluidine blue O staining).

quentially to maximize the number of cells in an extraction volume.

We compared the total RNA amounts recovered from cells selected from a variety of tissue sources, and we measured the proportionality between number of cells harvested and RNA recovery. The amount of RNA recovered varied by tissue source and cell type, but was consistently in the range of 10 to 50 ng RNA 100 cells^{-1}. This compares favorably with yields reported for captured animal cells (Mills et al., 2001). Figure 4B shows that the yield of RNA from maize BS cells (approximate diameter, 20 μm) and radish cotyledon cells (approximate diameter, 10 μm) was within this range and that the yield varied linearly with the number of cells harvested, but with a different slope for each. Yield from a particular cell type was reproducible over at least three experiments, suggesting that a cell type has constant properties relevant to cell harvest and RNA extraction. For example, we consistently recovered two to three times more total RNA from BS cells than from mesophyll cells harvested from the same tissue sections (data not shown). The yield differences between different cell types might be due to intrinsic biological differences, to differences in RNA extraction efficiency for different cell sources, or to a combination of these. This suggests that care should be used to normalize yields in designing experiments that rely on quantitative comparisons between different cell types.

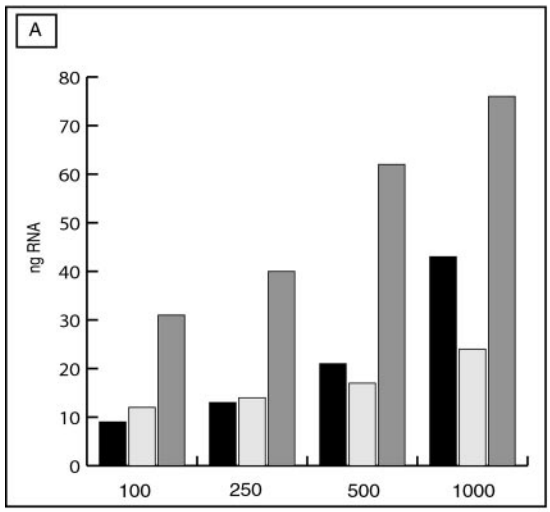
Specificity and Quality of RNA from Harvested Cells

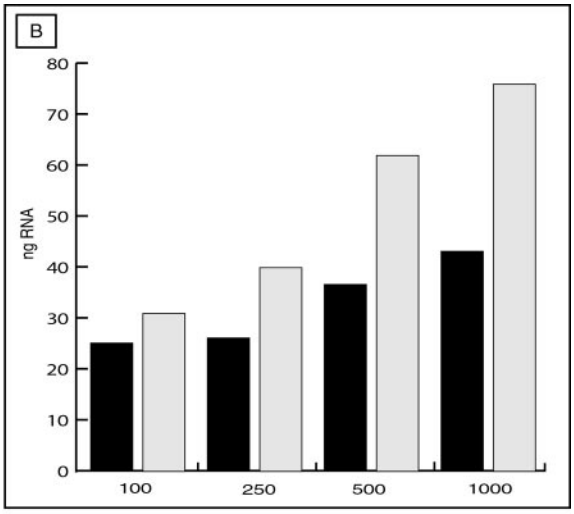
To evaluate the purity of cell types harvested by LCM, we captured adjacent BS and mesophyll cells from sections of maize leaves, isolated RNA, and analyzed it by reverse transcriptase (RT)-PCR for the presence of C4-specific NADP-malic enzyme (NADP-ME) and ubiquitin RNA (Fig. 5). RNA for ubiquitin is non-cell-specific and was used as a positive control (Christensen et al., 1992). NADP-ME has been demonstrated by in situ hybridization, preparative cell separations, and reporter gene expression to be accumulated in BS cells, but not in mesophyll cells, as a component of the C4 pathway (Sheen, 1999). Each of these methods has a limited ability to resolve signals between adjacent cells because of the limits of cell separation and probe detection methods. However, LCM has the potential to absolutely resolve one cell type from the other, because individual cells of one type or the other are removed from the tissue context.

We found that NADP-ME RNA was amplified only in the maize BS cell sample, whereas ubiquitin RNA was amplified in both BS and mesophyll cells (Fig. 5A). Three independent primer sets spanning intron regions for the C4-specific NADP-ME, generating products of 613, 791, and 965 bp, were used to confirm that the RT-PCR products were from BS cell RNA transcripts rather than from DNA (Fig. 5B). These included one set that produced a 965-bp product, which is approximately one-half the size of the spliced message. For these RNAs, approximately 50 to 150 harvested cells were required to obtain the approximately 5 to 10 ng of RNA needed for unambiguous RT-PCR signals from paraffin-embedded samples. In another test, 5 to 10 ng isolated from approximately 50 to 150 harvested Arabidopsis leaf mesophyll, pavement, or guard cells gave strong RT-PCR signals with primers for both actin2 and actin8, which have been shown to be transcribed in most cells (Fig. 5C and data not shown; An et al., 1996). In contrast, primers to the carbonic anhydrase, chloroplastic precursor, which is mesophyll specific in expression (Jacobson et al., 1975; Brandt et al., 2002), produced the expected 292-bp product only from RNA isolated from mesophyll cells (Fig. 5C).

For other cells and probes, the apparent cell specificity and sensitivity will depend on the abundance

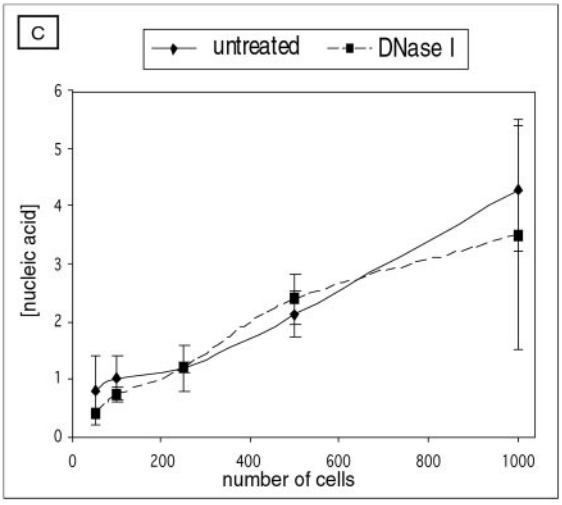
Figure 2. (*Legend continued from facing page.*)
Maize BS cells captured from two sectioned leaves. R through T, Capture of tomato shoot tip protoderm: R, before laser; S, tissue remaining after capture; and T, protoderm of leaf and shoot apical meristem captured on cap. U and V, Capture of abaxial and adaxial portions of tomato leaf primordium P1. U, tissue remaining after abaxial capture; inset, captured P1 abaxial tissue. V, Tissue remaining after adaxial capture following capture of abaxial leaf tissue; inset, captured P1 adaxial tissue. W, Laser capture of maize root meristem showing tissue remaining after capture of quiescent center; insets, quiescent center capture and surrounding proximal and distal meristem tissue). X through Z, Capture of rice seedling procambium: X, before laser; Y, tissue remaining after capture; and Z, procambium captured on cap.





number of cells	100	250	500	1000
Trizol	9 ± 4	13 ± 4	21 ± 4	43 ± 11
Stratagene	12 ± 6	14 ± 7	17 ± 8	24 ± 12
Picopure	31 ± 10	40 ± 2	62 ± 4	76 ± 1

number of cells	100	250	500	1000
Radish hypocotyl	31 ± 10	40 ± 2	62 ± 4	76 ± 1
Maize bundle sheath	25 ± 7	26 ± 5	36 ± 4	43 ± 20



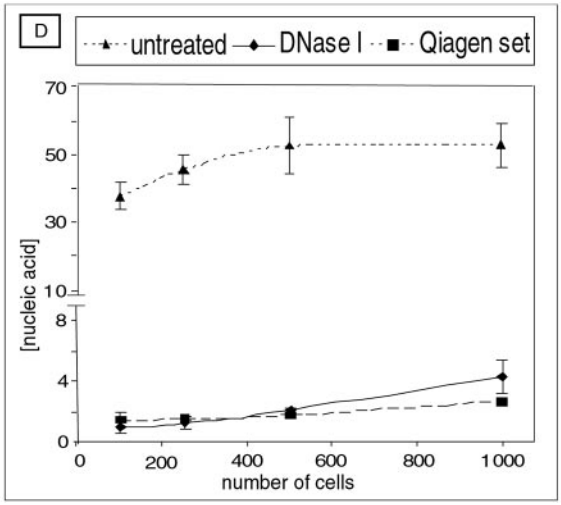


Figure 4. A, RNA recovery from radish hypocotyl cells using different methods: TRIzol (black bars), Miniprep kit (light gray bars; Stratagene, La Jolla, CA), and Arcturus Engineering PicoPure kit (gray bars). B, Comparison of RNA recovery from different cell types: radish hypocotyl (light gray bars) and maize BS cells (black bars). The tables below A and B show the means and SDs of three independent experiments. C, DNase treatment of RNA recovered using the TRIzol extraction. D, DNase treatment of RNA recovered using the PicoPure kit. Two treatments are shown: DNase I and DNase set (Qiagen USA, Valencia, CA). Note that the *y* axis is not continuous.

of the RNA in the target cells. We easily measured the relatively abundant RNAs for aquaporin, CDC2, phospho*enol*pyruvate carboxylase, malate dehydrogenases, and actin in cells captured from Arabidopsis, maize, and radish (data not shown). However, we found that individual low abundance messages in small-cell samples can vary between positive and negative RT-PCR signals. This stochastic behavior has been noted in a variety of high-sensitivity studies of transcription in one or a few cells, and it appears to be due to at least two biological phenomena. First, transcriptional regulation is generally not absolute. Genes judged by less sensitive methods to be transcriptionally inactive can produce enough transcripts

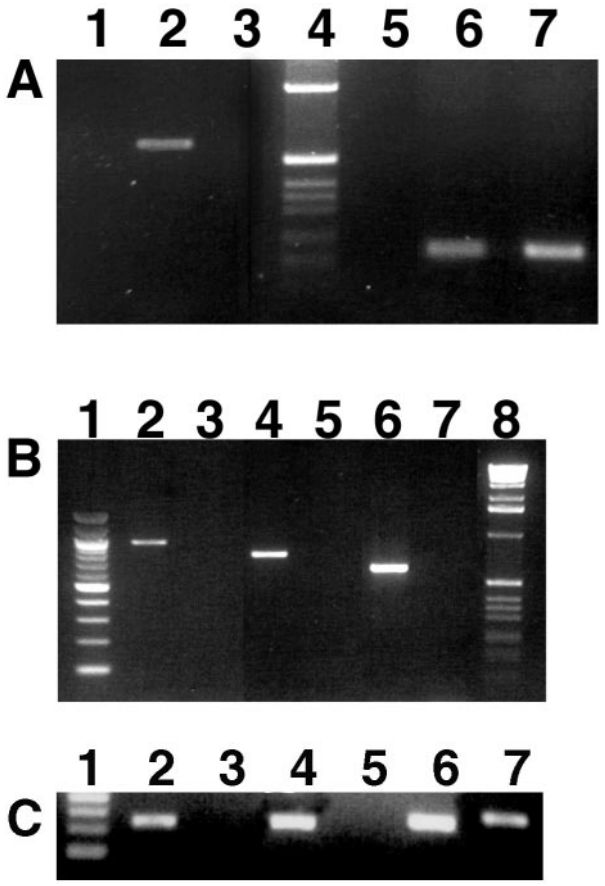


Figure 5. Specificity of RNA recovered from cells isolated by LCM. In A and B, maize RNA samples were subjected to RT-PCR using primers for ubiquitin (UBI, positive control) or NADP-ME, as described in "Materials and Methods." A, Lane 1, Mesophyll cells (ME primers [ML1415/1927L]; lane 2, BS cells (ME primers [same]); lane 3, water (ME primers [same]); lane 4, 1-kb DNA ladder; lane 5, water (UBI primers [MUBI1–5/MUBI1–6]); lane 6, mesophyll cells (UBI primers [same]); lane 7, BS cells (UBI primers [same]). B, Lane 1, 100-bp ladder; lanes 2, 4, and 6, BS cells; lane 3, 5, and 7, water; lanes 2 and 3, ME primers MEFOR/PMEL; lanes 4 and 5, ME primers 1350C/RTPMEL; lanes 6 and 7, ME primers ML1415/1927L; and lane 8, 1-kb DNA ladder. C represents RT-PCR reactions from Arabidopsis cells using primers for actin8 (positive control) or carbonic anhydrase, chloroplastic precursor (At3g01500, mesophyll-specific). C, Lane 1, 100-bp ladder (the lowest band is 100 bp); lanes 2 and 3, RNA from stomatal cells; lanes 4 and 5, RNA from pavement cells; and lanes 6 and 7, RNA from mesophyll cells. Even lanes (2, 4, and 6) were amplified with primers AACT8F and AACT8R, and odd lanes (3, 5, and 7) were amplified with primers CACPF and CACPR. All panels are photos of single gels, from which unloaded spacer lanes have been removed, without any other realignment.

to be detectable by methods that greatly amplify transcripts from few cells (Chelly et al., 1989; Sarkar and Sommer, 1989). Signals from such genes will be detected stochastically, depending on the sensitivity and noise level of the detection method. Second, populations of cells with apparently uniform transcriptional behavior, such as a particular cell type, are in fact made up of cells in a variety of transcriptional states for which the population exhibits an average behavior (Levsky and Singer, 2003). The smaller the sample from that population, the more likely a deviation from average will be captured at a particular gene. This probabilistic behavior of few cells was recently observed among plant cells captured by micropipetting (Brandt et al., 2002). If it is necessary to work with samples of very few cells (e.g. 25 or less from paraffin-sections), it is important to perform multiple replicates or to pool a substantial number of samples. These threshold issues should have less impact in quantitative assays of transcript levels such as quantitative real time-PCR and microarray hybridization.

Methodologies such as microarray analysis currently require microgram amounts of a probe that uniformly represents the RNA population. LCM from tens of cells provides nanogram or picogram amounts of RNA, and therefore requires a high-efficiency linear amplification to produce such probes. To evaluate the general quality of the small amounts of RNA isolated from LCM-isolated cells, we subjected samples of RNA corresponding to approximately 10 radish cortical parenchyma cells (2.5 ng of total RNA) to one or two rounds of linear amplification by T7 transcription of cDNA (Table I) and separated the products by gel electrophoresis (data not shown). As expected, the first round produced 36 ± 9 ng of RNA and the second round produced 2.15 ± 0.51 μg of RNA. This is easily enough RNA to serve as a microarray probe, because the amplified product corresponds to mRNA only, whereas microarray probes are generally labeled from total RNA. The resulting products were distributed in a range of sizes from about 500 to larger than 2,500 bases, similar to the distribution generally observed in large scale poly(A^+) RNA preparations from most plant sources (data not shown). Our own tests comparing amplified and unamplified Arabidopsis RNA probes on Arabidopsis microarrays (L. Ma, X.-W. Deng, T. Ceserani, and T. Nelson, unpublished data) confirmed the manufacturer's quality control tests of the linearity of amplification (our unamplified/amplified correlation coefficient = 0.92 for one round, 0.87 for two rounds). This suggests that it is feasible to harvest the required number of cells for microarray experiments requiring microgram amounts of RNA, even for cell types with lower RNA yields with the LCM method described here.

Table I. *Linear amplification of mRNA from LCM-captured cells*

2.5 ng of total RNA from LCM-captured radish cortical parenchyma cells was subjected to one or two rounds of amplification, as described in "Materials and Methods." Data are averages of four independent experiments.

Total RNA at Start	mRNA after First Round	mRNA after Second Round
	ng	
2.5	36 ± 9	2,147 ± 509

DISCUSSION

LCM is a method applicable to plant tissues embedded in paraffin, making feasible the analysis of DNA, RNA, and protein from cells selected from microscope sections with an appearance familiar to most plant biologists. This should provide a means of linking many historical and ongoing studies of cellular relationships and roles to comprehensive genomic and proteomic datasets.

MATERIALS AND METHODS

Tissue Preparation

Tissue samples were trimmed to 4 mm or less in thickness and fixed 4 to 24 h (depending on thickness) at 4°C in at least 10 volumes of freshly prepared 3:1 ethanol:acetic acid (Farmer's fixative) or formalin-acetic acid-ethanol according to Ruzin (1999) or in Prefer (Anatech, Ltd., Battle Creek, MI) for 8 to 10 h at 4°C. Prefer is a cross-linking formalin-free fixative. When needed, tissue in fixative was subjected to 15 min of vacuum to assist sinking and infiltration. Fixed tissue was dehydrated at room temperature in a graded series of ethanol (3 h each [v/v] 75%, 85%, 100%, 100%, and 100%), followed by an ethanol:xylenes series (3 h each [v/v] 75%:25%, 50%:50%, 25%:75% 0%:100%, 0%:100%, and 0%:100%). Flakes of Paraplast-X-Tra tissue embedding medium (Fisher Scientific, Fair Lawn, NJ) were added to the final step. Once the flakes dissolved at room temperature, liquefied Paraplast-X-Tra was added, and sample vials were transferred to an oven at 58°C. The medium was replaced at 3- to 6-h intervals until the odor of xylenes was absent. Samples were positioned in Paraplast-X-Tra, and sections were cut on a rotary microtome (Microtom HM310, Waldorf, Germany), floated in water on Probe-on microscope slides at 42°C to stretch ribbons, air-dried, and stored in darkness at 4°C under dehydrating conditions. For LCM, slides were deparaffinized in xylenes for two changes of 5 min and air-dried.

LCM

The Pix-Cell II LCM system was used to microdissect cells from deparaffinized and dried tissue sections prepared as above. The laser beam was adjusted to melt the thermoplastic film in a spot of a diameter that visually corresponded to the diameter of the target cell. Captures were performed using 7.5- or 15-μm beam diameters according to cell size. Power settings were 50 or 40 mW, and laser pulse durations were 650 μs and 2.5 ms, respectively. The success of harvest was evaluated by comparison of the image of cells captured on the cap versus the image of the tissue after removal of harvested cells. In general, the targeted plant cells were precisely removed from the section to the cap film, with nearly 100% efficiency, and without visible contamination with other cells. If additional material adhered to the harvested cells, they could be removed by blotting the post-harvest film with a Post-It adhesive strip (3M, St. Paul). Only cells immediately within the laser halo adhere to the thermoplastic film, and other cells are removed onto the Post-It strips. This treatment did not appear to influence the yield or quality of subsequent RNA extractions.

RNA Extraction

For the TRIzol method, caps with captured cells were fitted to 0.5-mL Eppendorf tubes containing 300 μL of TRIzol reagent (Invitrogen), and the tubes were inverted and stored at −80°C or extracted immediately. The samples were thawed if necessary and incubated at room temperature for 5 min with intermittent vortexing. The caps were removed, 60 μL of chloroform was added, and the tubes were shaken for 15 s. After 3 min of incubation at room temperature, the tubes were spun at 12,000*g* at 4°C for 15 min. The resulting upper phase was transferred to a new tube, and 20 μg of glycogen (Sigma-Aldrich, St. Louis) was added as a carrier along with 150 μL of isopropanol. The tubes were mixed well and incubated either for 10 min at room temperature or for 20 min at −80°C, and then spun at 12,000*g* at 4°C for 30 min. The resulting pellet was washed with 75% (v/v) ethanol, and the tubes were respun at 7,500*g* at 4°C for 5 min. After removing the ethanol, the pellet was air-dried for 10 to 15 min at room temperature. The RNA was resuspended in 10 μL of RNase-free water and RNase inhibitor (RNAguard, Amersham Biosciences, Uppsala) was added to a final concentration of 2 units μL^{-1}. This method was amenable to combining cells captured on several caps into a single extraction. For the PicoPure kit (Arcturus Engineering) and the RNA Miniprep and Nanoprep kits (Stratagene), we followed the manufacturers' instructions.

DNase I Treatment

To eliminate DNA from aqueous RNA extractions, samples of isolated nucleic acid were treated with 10 units of RNase-free DNase I (Roche Diagnostics, Mannheim, Germany) in 50 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, and 50 $\mu g\ mL^{-1}$ RNase-free bovine serum albumin for 30 min at 37°C. RNA isolated by the PicoPure method was routinely treated with the RNase-Free DNase Set kit (Qiagen USA) according to the manufacturer while the samples were on the PicoPure column membrane, incubating for 15 min at room temperature before eluting the column.

RNA Quantification

RNA was measured fluorometrically on a microtiter plate reader (Wallac 1420, PerkinElmer Wallac, Turku, Finland). Samples were measured after 2 min and 5 min of incubation with the Ribogreen reagent (Molecular Probes, Eugene, OR) in black microtiter plates, with 485 nm excitation and 535 nm emission wavelengths, according to the manufacturer.

RT-PCR

RNA was reverse-transcribed using Sensiscript RT (Qiagen USA), primed with 1 μM oligo(dT) (18-mer, Ambion, Austin, TX) or 0.25 μM specific primer. PCR was performed with the Readymix RedTaq PCR Reaction Mix (Sigma-Aldrich), with 1 pmol μL^{-1} of specific primers. The PCR conditions were adjusted based on the primers used. Primers were as follows.

Ubiquitin primers

MUBI1-5 (5′ggtggtatgcagatctttg3′) and MUBI1-6 (5′gtagtctgctagggtgcg3′) for 182 bp product

BS-specific ME primers

MEFOR (5′caggttgttagcagcactcaag3′) and PMEL (5′caatgcctctccagcagcacc3′) for 965 bp product; 1350C (5′cgctccaattgaagagtgcgcaag3′) and RTP-MEL (5′cagggactataaacaacagagtac3′) for 791 bp product; ML1415 (5′ggctccttcagccattcaag3′) and 1927L (5′cggtagacgggagtgtacatg3′) for 613 bp product

Actin8 primers(Laval et al., 2002)

AACT8F (5′ctaactaaagagacatcgtttcca3′) and AACT8R (5′gtttttatccgagtttgaagaggct3′) for 250 bp product

Carbonic anhydrase primers

CACPF (5′gacttcatagaggactgggtc3′) and CACPR (5′aatgtagtatggtagccacatc3′) for 292 bp product.

RNA Amplification

A T7 polymerase-based linear amplification systems, RiboAmp (Arcturus Engineering), was used according to the manufacturer. This proprietary system relies on forming double-stranded cDNA, followed by in vitro transcription by T7 polymerase. The manufacturer's quality control tests include the testing of amplified and unamplified probes on microarrays. The amplified probe signal matches the original RNA source, with a correlation coefficient of 0.91; duplicate amplifications have a reproducibility of $r = 0.99$ (http://www.arcturus.com). This is consistent with our own tests of the amplification system (see "Results"). Amplified RNA products were measured by RiboGreen fluorescence, as described above, and were run on a 1% (w/v) formaldehyde gel followed by staining in 1× SYBR Gold gel stain (Molecular Probes) to estimate the range of product sizes.

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