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• BASIC RESEARCH •

Gene-expression analysis of single cells-nested polymerase chain reaction after laser microdissection

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Abstract

AIM: The structural and functional characteristics of cells are dependent on the specific gene expression profile. The ability to study and compare gene expression at the cellular level will therefore provide valuable insights into cell physiology and pathophysiology.

METHODS: Individual cells were isolated from frozen colon tissue sections using laser microdissection. DNA as well as RNA were extracted, and total RNA was reversely transcribed to complementary DNA (cDNA). Both DNA and cDNA were analyzed by nested polymerase chain reaction (PCR). The quality of isolated DNA and RNA was satisfactory.

RESULTS: Single cells were successfully microdissected using an ultraviolet laser micromanipulator. Nested PCR amplification products of DNA and cDNA of single cells could clearly be visualized by agarose gel electrophoresis.

CONCLUSION: The combined use of laser microdissection and nested-PCR provides an opportunity to analyze gene expression in single cells. This method allows the analysis and identification of specific genes which are involved in physiological and pathophysiological processes in a complex of variable cell phenotypes.

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INTRODUCTION

Techniques for isolating a specific cell population from a tissue complex for subsequent analysis of its molecular and biochemical contents have long been critical in cellular and molecular biology. To this end, various microdissection techniques have been developed to reduce contamination of surrounding cells[1-3]. Microdissection originally involved manual or micromanipulator guidance of a needle to scrape off an area of interest of a thin tissue section[4]. Selective ultraviolet radiation fractionation, which relies on negative selection and ablation of the unwanted areas of the tissue on the slide, provides a technical advancement in this field[1]. Micromanipulators and microdissection have improved the accuracy and reliability of microdissection; however, it remains an intrinsically slow, technique-dependent process of procuring pure cell populations from tissues. Modern techniques, such as flow cytometry with cell sorting and affinity-labeled magnetic beads, allow separation of cell subpopulations from heterogeneous pools of single cells in suspension. To apply these techniques to tissues, there is a requirement for the dissolution of intercellular adhesion and the formation of a suspension of individual cells, which is not generally practical in solid tissues and may change the characteristics of the isolated cells. Perhaps the biggest breakthrough in this approach and one that is rapidly gaining popularity is laser microdissection (LM)[5,6]. LM can be used to collect individual cells or specific cell populations from complex tissues without any contamination, and an individual operator can collect many samples in a single session.

The use of LM to obtain pure cell populations has so far been applied to DNA analysis[5], protein analysis[7] and mRNA analysis[8]. A variety of approaches are routinely used to assess the expression of specific genes in cells and tissues, such as Northern blot and RNase protection assay. The quantity of mRNA that can be harvested from a single cell is on the order of 1 pg at best. Thus, the techniques used to analyze gene expression are limited when applied to single cells. Nested PCR has proved to be a sensitive and specific procedure[9], and the use of nested PCR increases both the sensitivity and specificity of the standard PCR assay[10,11].

We now present an approach that allows analysis of DNA and mRNA down to the cellular level within intact tissue sections using a combination of LM and nested PCR.

MATERIALS AND METHODS

Preparation of tissue sections

Normal colon tissues were obtained from operation specimens in which a partial colon resection was performed for colon cancer. The Human Subject Committee of the University of Bern approved the studies. Immediately following surgical removal, tissues were snap-frozen in liquid nitrogen and maintained at -80 ℃ until use. Tissues were embedded in Tissue Tek OCT medium (VWR Scientific Products Corporation, San Diego, CA, USA) and sectioned at 8 μm in a cryostat, mounted on uncoated glass slides, and immediately stored at -80 ℃ once air dried. Slides containing frozen sections were fixed in 70 % ethanol for 2 min, stained with hematoxylin and eosin, then dehydrated in 70 %, 94 % and 100 % alcohol (each for 2 minutes) and finally dehydrated for 2 minutes in xylene.

Laser microdissection

The ultraviolet-laser Robot-Microbeam (P.A.L.M., Wolfratshausen, Germany) used for microdissection consists

of a nitrogen laser of high-beam precision (wavelength 337nm) coupled to an inverted microscope (Axiovert 135; Zeiss, Jena, Germany) via the epifluorescence illumination path. The microscope stage and micromanipulator are digitally controlled and moved by a computer mouse. The high photon density within the laser focus catapults the material to the cap without any heating effect-a so-called cold ablation-so that DNA and RNA would not degrade during microdissection. With the combination of laser-manipulated microdissection (LMM) and the laser pressure catapulting (LPC) technique, single cells could be processed in seconds[12].

DNA extraction from microdissected samples

DNA was extracted using DNA extraction solution with 100 mM Tris-HCl, pH 8.0, 400 μg/ml proteinase K (Sigma, Deisenhofen, Germany). After incubation at 37 ℃ for 3 h, the samples were boiled to inactivate proteinase K. After centrifugation, the supernatants of the DNA extraction buffer, now containing DNA from the microdissected cells, were used for nested PCR.

RNA isolation from microdissected samples and reverse transcription

Total RNA was independently isolated by means of a modification of the RNA microisolation protocol, as described previously[13]. Briefly, caps were placed in Eppendorf tubes containing guanidinium isothiocyanate buffer, inverted several times, extracted with phenol/chloroform/isoamyl alcohol, and precipitated with sodium acetate and glycogen carrier (10 μg/μl) in isopropanol. After initial recovery and resuspension of the RNA pellet, a DNAse treatment was performed for 2 h at 37 ℃ using 10 units of DNAse (Roche Diagnostics, Mannheim, Germany) in the presence of 10 units of RNAse inhibitor (Roche Diagnostics, Mannheim, Germany), followed by re-extraction and precipitation. The pellet was resuspended in 24 μl of RNAse-free water. 12 μl of total RNA was reversely transcribed into complementary DNA (cDNA) using random hexamers according to the manufacturer' s instructions (Roche Diagnostics, Rotkreuz, Switzerland)[14]. For each cDNA reaction tube, an identical tube containing the same amount of RNA was prepared as a negative control (mock RT). In these tubes, the same amount of water was substituted for reverse transcriptase. After incubation, the reaction was terminated by heating to 95 ℃ for 10 min. The cDNA preparations were used immediately or stored at -20 ℃ until use.

Gene analysis by nested PCR

The human beta-actin gene, a ubiquitously and constitutively expressed gene, was used as the target gene[15] for nested PCR. This approach involves the use of two pairs of PCR primers. The primers were synthesized by Amplimmun (Amplimmun AG, Madulain, Switzerland); the sequence is shown in Table 1. PCR amplification was carried out using beta-actin-outer primers in a final volume of 25 μl with a Perkin-Elmer GeneAmp System 9700 using 0.625U of Taq DNA polymerase (Roche Diagnostics GmbH, Mannheim, Germany). Cycling conditions were as follows: 35 cycles of denaturation at 94 ℃ for 1 min, annealing at 62 ℃ for 1 min, and elongation at 72 ℃ for 2.5 min. The first PCR cycle was preceded by denaturation at 94 ℃ for 3 min, and the last PCR cycle was followed by incubation at 72 ℃ for 8 min. Nested PCR was performed using 0.5 μl of the first PCR product as a template. The PCR cycling conditions for the beta-actin-inner primers were the same as above except for an annealing temperature of 60 ℃. The amplification products were analyzed by electrophoresis on 1 % agarose gels and stained with ethidium bromide.

Table 1 β-actin primers used for nested PCR analysis

Primer	Sequence	Primer size (bp)	Size of PCR products (bp)
β-actin-outer			
Forward primer	GGC ATC CTC ACC CTG AAG TA	20	494
Reverse primer	CCA TCT CTT GCT CGA AGT CC	20	
β-actin-inner			
Forward primer	AAA TCT GGC ACC ACA CCT TC	20	240
Reverse primer	AGG GCA TAC CCC TCG TAG AT	20	

RESULTS

Laser microdissection of cells from cryostat sections

Eight-micrometer cryostat sections were prepared from normal colon tissues. Single colon mucosa cells were selected and cut with LM and catapulted by LPC under visual control (Figure 1). For LPC, the setting of laser energy was sufficiently high to catapult the microdissected cells into the microcentrifuge cap. Cell clusters of interest were also selected and laser-microdissected under visual control (Figure 2). The laser precisely circumcised a selected area or a single cell, which yielded a clear-cut gap between selected and non-selected areas.

DNA analysis in microdissected cells by nested PCR

100 cells, 10 cells and 1 cell were microdissected from normal colon sections. Samples were digested by proteinase K and boiled for 10 min to denature proteinase K. After centrifugation, the supernatants were used for PCR with the beta-actin-outer primers. The amplification products were analyzed by electrophoresis on 1 % agarose gels; the results are shown in Figure 3A. The bands from a complete section, 100 cells, and 10 cells could be clearly visualized. Nested PCR was performed using 0.5 μl of the first PCR product as a template; the amplification results are shown in Figure 3B. Amplification products from single cells could be clearly visualized by agarose gel electrophoresis after nested PCR. Control amplifications without DNA templates did not yield any signal.

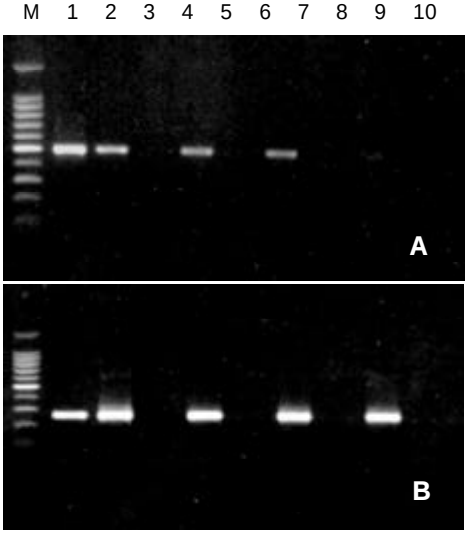

Figure 2 Amplification results from nested PCR of colon cells after laser microdissection. Nested PCR amplification products of DNA of single cells could clearly be visualized by agarose gel electrophoresis. A: Amplification results of β-actin-outer primers. B: Amplification results of β-actin–inner primers. M: DNA markers (upper to lower: 2000, 1000, 900, 800, 700, 600, 500, 400, 300, 200, and 100 bp). 1: PCR positive control; 2: one complete section; 4, 6 and 8 are 100, 10 and 1 cell(s), respectively; 3, 5, 7 and 9 are negative controls of 2, 4, 6 and 8; 10: PCR negative control.

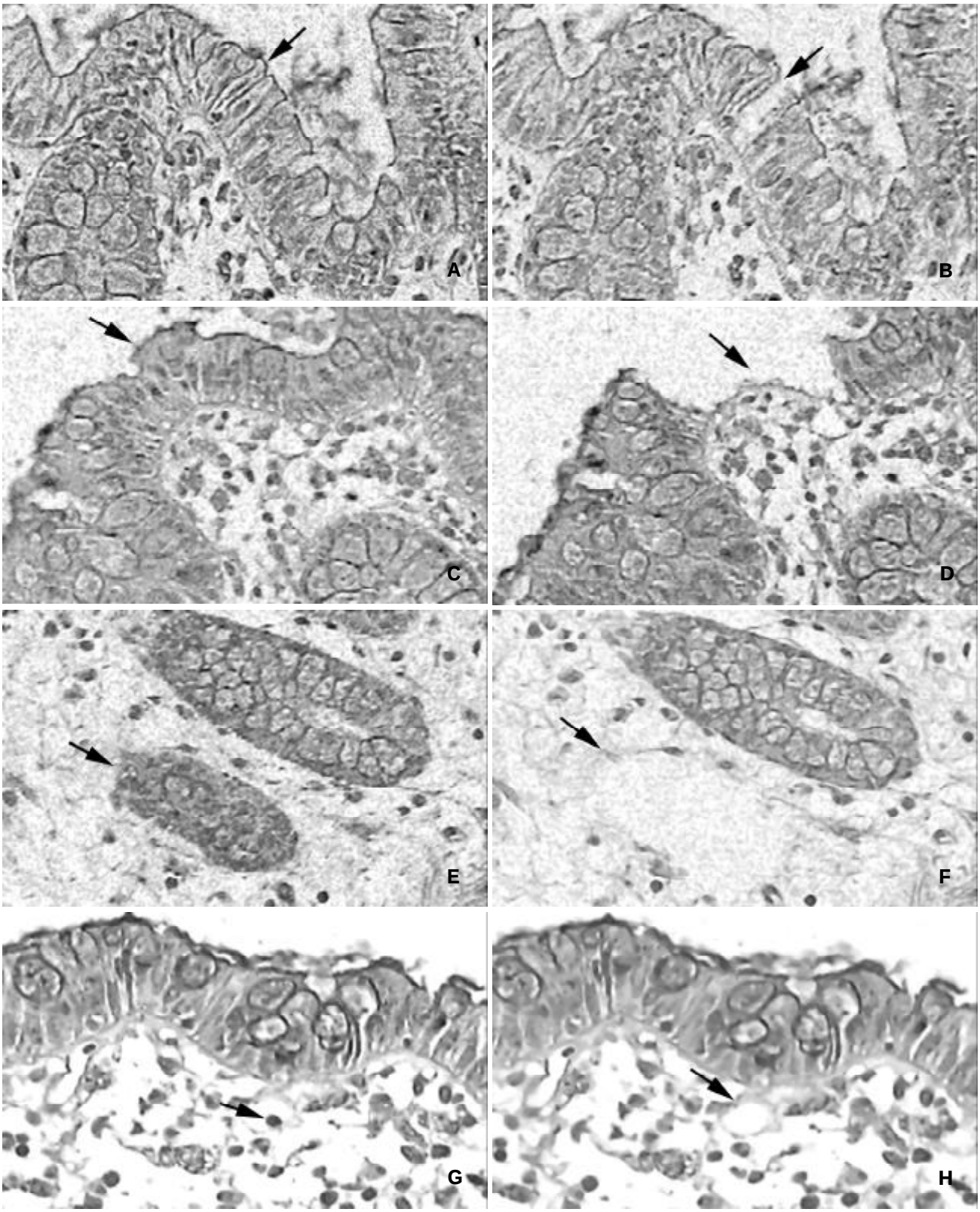

Figure 1 Examples of laser microdissection in colon tissues (hematoxylin and eosin, magnification ×200). Single cells were picked by combination laser-manipulated microdissection (LMM) and laser pressure catapulting (LPC). A, C, E, G: Before Laser microdissection. B, D, F, H: Using LMM, the laser precisely circumcised selected cells, yielding a cut gap between selected and non-selected areas. Then the selected cells were catapulted using the LPC technique. A-F: Typical images before and after LMM and LPC. G, H: Laser microdissection could also be used to cut out the nucleus of a selected cell.

mRNA expression in microdissected cells by nested PCR

Normal colon RNA was obtained from complete sections, 200 cells, 100 cells, 10 cells and single cells. cDNA was transcribed from total RNA. The amplification products were analyzed by electrophoresis on 1 % agarose gels; the results are shown in Figure 3C. Signals from complete sections could be clearly visualized. However, the signals from 200 cells and 100 cells could only be seen on the original gels, and the signals from 10 cells and single cells were below the level of detection. Nested PCR was performed using 0.5 μl of the first PCR product as a template; the amplification results are shown in Figure 3D. Amplification products of even single

cells could be clearly visualized by agarose gel electrophoresis. Control amplifications without cDNA templates did not yield any signal.

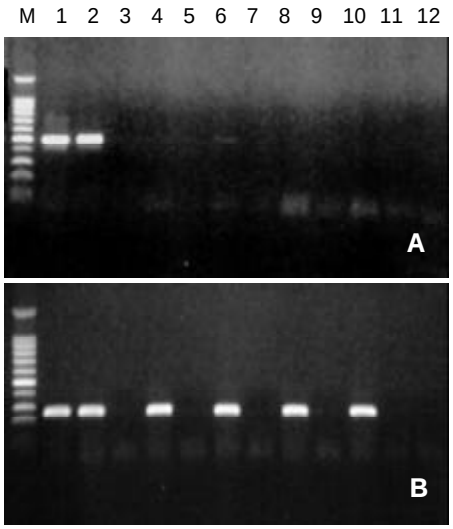

Figure 3 Amplification results from nested RT-PCR for cDNA of single cells after laser microdissection. A: Amplification results of β-actin-outer primers. B: Amplification results of β-actin-inner primers. M: DNA markers (upper to lower: 2000, 1000, 900, 800, 700, 600, 500, 400, 300, 200, and 100 bp). 1: PCR positive control; 2: one complete section; 4, 6, 8 and 10 are 200, 100, 10 and 1 cell(s), respectively; 3, 5, 7, 9 and 11 are negative controls of 2, 4, 6, 8 and 10; 12: PCR negative control.

DISCUSSION

In this study, single colon mucosa cells could be selectively microdissected and collected from frozen tissue sections under direct microscopic visualization using LM. Microdissected mucosa cells were used for further gene analysis using nested PCR.

Most tissues are composed of a number of different cell types, and cellular heterogeneity in most tissues is very complex. In normal or developing organs, specific cells express different genes and undergo complex molecular changes both in response to internal control signals, signals from adjacent cells, and humoral stimuli. In disease pathologies, the diseased cells of interest, such as precancerous cells or invading groups of cancer cells, are surrounded by these heterogeneous tissue elements. The percentage of non-neoplastic cells present in such specimens can be as high as 95 %[16]. This tissue heterogeneity has proved one of the major obstacles to molecular research using current methods, since varying numbers of normal cells could mask the presence of single abnormal cells when analysis of its gene expression and total DNA or RNA from heterogeneous tissue is used[17-24].

LM is a new method for performing microdissection of selected regions down to a single cell[5,6,25]. There have been other reports describing methods to collect specific regions of tissue, such as graded sieving of glomeruli or pancreatic islet cells[26,27] and isolation of suspension of proximal tubular cells[28]. These methods make it possible to obtain relatively pure and large amounts of samples; however, it is difficult to avoid possible contamination of other tissue compartments, which can cause problems with sensitive RT-PCR methods. Using LM, our study has shown that it is feasible to examine mRNA expression of single cells without the risk of contamination by neighboring cells. If more than one cell is needed, single unwanted cells can selectively be destroyed from the tissue section using LM, resulting in an area composed of a homogenous cell population. Utilizing a computer-controlled microscope stage, the cells of interest are visually selected and marked and their positions are stored electronically. The stage then automatically goes to and moves around the selected cells, while the laser fires with a preselected pulse energy and repetition rate. With a further slightly defocused laser shot, the target cells can selectively be catapulted into a microcentrifuge cap using LPC for further study. As reported in this article, this method allows gene analysis in the particular tissue region isolated from the frozen cryostat specimen, avoiding the contamination of other cells.

The quantity of mRNA that can be harvested from a single cell is approximately 1 pg at best. Therefore, to obtain meaningful gene-expression data, well-optimized or specialized amplification protocols must be applied. Using conventional PCR, the theoretical limit of detection is one copy of a single-stranded DNA molecule, and so with efficient harvesting of cytoplasm and a well-optimized PCR protocol, single-cell PCR is feasible[29]. However, this is not a trivial undertaking, and identifying the expression of rare or particularly labile transcripts would prove to be technically demanding[30].

In this study, amplification signals from DNA of single cells were only faintly present in the agarose gels, and amplification signals from cDNA could not be visualized by gel electrophoresis. Therefore, certain modifications to this approach have to be applied to provide more comprehensive single-cell expression analysis. In this study, a straightforward method of expanding the results obtained from single-cell PCR involves the use of nested PCR. Essentially, a primary conventional PCR increases the target concentration so that a second PCR reaction can be carried out to assay for the presence or absence of gene expression. The present study has shown that nested PCR is feasible for the analysis of gene expression after LM, even at the cellular level, and that this approach has the advantage of being relatively simple to apply. Further studies on the gene expression profiles of single microdissected cells will provide novel insight into different physiological and pathophysiological processes.

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