Live cells from cell culture: Direct isolation of poly-A(+) mRNA from individual laser microdissected cell samples obtained with the PALM® MicroBeam system

GABRIELE FRIEDEMANN, Ph.D.,
P.A.L.M. Microlaser Technologies AG,
Am Neuland 9 + 12, 82347 Bernried, Germany

KEY WORDS: live cells, cell culture, mRNA, microdissection, LMPC, single cell, QuickPick™, PickPen®

ABSTRACT

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 provide valuable insights into cell physiology and is useful in a wide range of research and clinical applications. Laser microdissection and pressure catapulting (LMPC; PALM® MicroBeam System) combined with real-time PCR for expression profiling allows several investigations of individual cells. The PickPen® technology together with QuickPick™ mRNA nano kit is engineered to recover high-quality mRNA consistently from 1 to 1000 cells very rapidly. Using LMPC and real-time PCR in combination with the PickPen® 1-M tool and QuickPick™ mRNA nano kit, we analyzed the expression of human porphobilinogen deaminase (PBGD) gene as a model system from 50, 10, 5 and 1 microdissected cells.

INTRODUCTION

Laser assisted microdissection is a well known technology to capture single cells for subsequent molecular analysis. LMPC is also used to catapult live cells from a cell culture for subsequent re-culturing, which is a convenient method to clone cells. The collection of single live cells directly out of a mixed culture avoids dilution series and allows to culture real single cells without the necessity to apply techniques of selective stimulation.
A new approach is mRNA preparation from single captured living cells for subsequent expression analysis.

A UV-A-laser mediated process dissects selected specimen and transfers them totally contact free directly into collecting vessels for subsequent analysis. A primary limitation of any target design is the amount of total RNA or poly-A(+) mRNA that can be obtained from limited amounts of cells. The recent developments in mRNA isolation techniques make previously time-consuming mRNA isolations attractive and practical. The conventional methods utilizing column filtration are not very suitable for working with less than 100 cells, as substantial amounts of mRNA may be lost in the resin-bed. The combination of pure cell preparation by LMPC, QuickPick™ mRNA kits, and PickPen® magnetic tools is a very potential instrument for rapid isolation of poly-A(+) mRNA from small sample amounts. The combination of both techniques is a famous tool in cell biology. High quality RNA can be extracted even from one single cell in a very fast and convenient way. Rapid handling results in less RNA degradation as well as higher RNA concentration.

No useful cells of a cell culture have to be squandered. One cell or a few cells are catapulted for further analysis. The remaining cells can be kept in culture and used for further experiments at a later date, which may be extremely helpful for the work with slowly growing and valuable cells.

PRINCIPLE OF THE QuickPick™ mRNA KIT

The QuickPick™ mRNA method is based on the binding of poly-A(+) mRNA to immobilized oligo $(dT)_{30}$ on paramagnetic particles. The sample is first lysed in Lysis/Binding Buffer. Washed magnetic particles are added to the lysed sample, followed by a 4-minute incubation at room temperature where the poly-A(+) hybridizes to the oligo $(dT)_{30}$ on the particles. The incubation is followed by three quick washes to remove impurities, such as pre-mRNA, rRNA, tRNA and snRNA. Finally the particles are transferred into the Elution Buffer. The mRNA still attached to magnetic particles is used directly as a solid-phase template in the RT-PCR reaction.

MATERIALS & METHODS

Cell culture
Bladder carcinoma cells (cell line EJ 28) were disseminated into a PALM® DuplexDish. The Duplex Dish consists of two membranes, a teflon membrane and the LMPC membrane. Only the LMPC membrane is cut and catapulted by the laser, so the surplus of non-catapulted cells can be put back into the incubator to wait for further experiments. The cells were grown in RPMI 1640 medium containing 10% Fetal Calf Serum and 10.000 U Penicillin/Streptomycin per 500 ml medium; they were incubated for 2 days at 37°C and 5% CO2.

Laser microdissection
Laser microdissection was performed using a PALM® MicroBeam System. Prior to LMPC (Laser Microdissection and Pressure Catapulting) the culture medium was removed and the cells of interest were circumscribed using a software tool. After laser cutting the desired cells were catapulted into a PALM® AdhesiveCap.

mRNA isolation
The collected cells were incubated in lysis buffer for a few minutes at room temperature and then vortexed for 20 seconds. The subsequent protocol was performed according to the QuickPick™ mRNA nano kit instructions. Particle-bound mRNA was resuspended in 5 µl elution buffer and used directly as a solid-phase template.

Reverse transcription
Resuspended particle-bound mRNA was reversely transcribed with the First Strand cDNA Synthesis Kit (Roche) by AMV-RTase. Random primers were used in a total volume of 20 µl for one hour at 42°C according to the manufacturer's protocol.

Real time PCR
PCR amplification of the cDNA was performed in a LightCycler® instrument (Roche). Reaction volume was 20 µl using protocols and components of the LightCycler-FastStart DNA Master^{PLUS} SYBR Green I Kit (Roche). We used 5 µl of each cDNA solution as template. cDNA specific primers für human PBGD gene were used as a model system producing a PCR-fragment of 158 bp.

RESULTS

Individual living cells cultivated in a DuplexDish are easily and reliably dissected and collected with the PALM® MicroBeam system (figure 1).

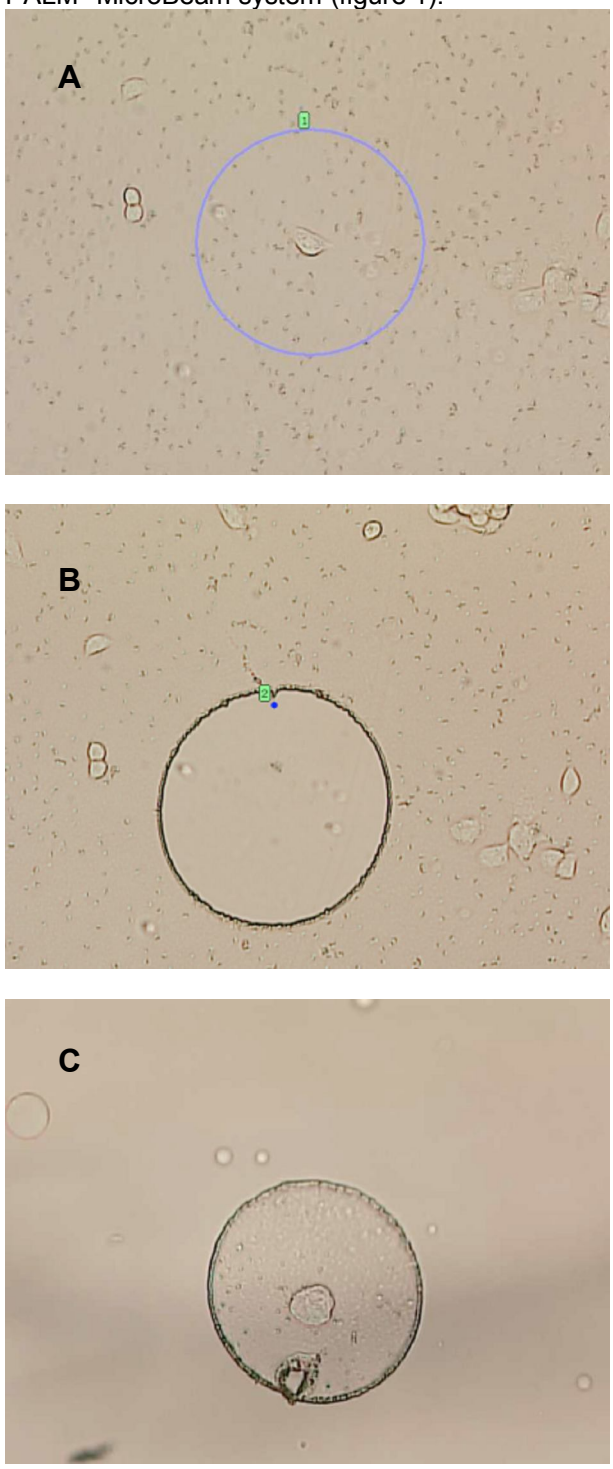

Figure 1: LMPC of a single live cell
A) cell circumscribed
B) cell after catapulting; note the hole in the LMPC membrane and the intact teflon membrane

C) single cell on the LMPC membrane in the collection device

50 cells, 10 cells, 5 cells and 1 cell, resp., were catapulted into clear PALM® AdhesiveCaps and analyzed subsequently. To exclude contamination errors we checked pure medium and empty membrane. 10 µl of pure culture medium and a piece of LMPC membrane from the Duplex Dish corresponding to the area of 10 cells, resp., were used as negative controls. After mRNA isolation samples were reverse and subsequently real-time PCR with the LightCycler® was performed using only a quarter of the cDNA reaction product as template.

The crossing points of the growth curves correlated very well with the number of cell equivalents used as templates (figure 2a).

The specificity of the PCR fragment was analyzed by Lightcycler melting curves (figure 2b).

Membrane areas incubated in culture medium without cells or pure culture medium never yielded specific PCR products (figure 2a and 2b).

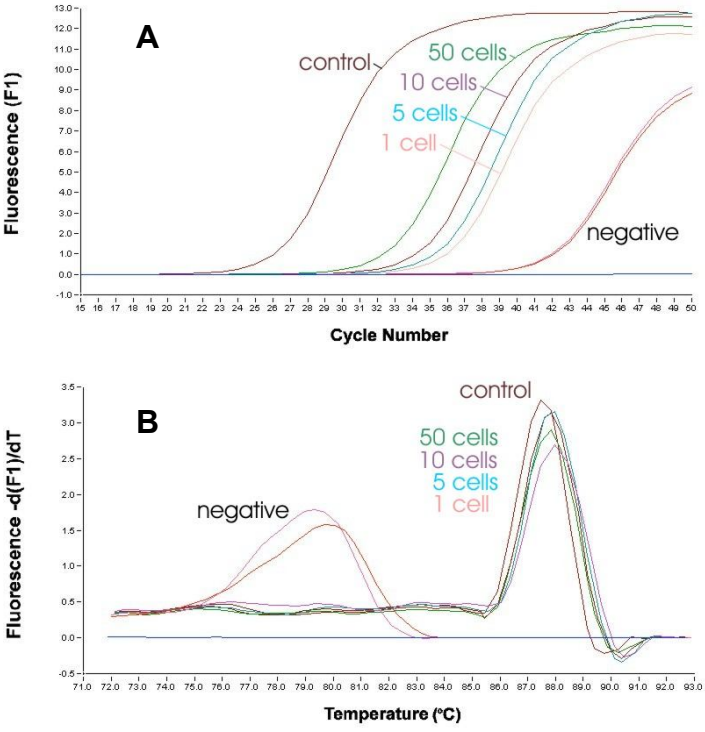

Figure 2: LightCycler analysis showing expression of the human PBGD gene.

A) LightCycler CT values correspond with the number of microdissected cells
B) specific melting curves prove the specificity of the PCR products

Green 50 cells; violet 10 cells; blue 5 cells; pink 1 cell

CONCLUSION

PALM® MicroBeam in combination with the PickPen® magnetic tool and QuickPick™ mRNA kits is a famous tool to study the expression profiles from individual living cell samples. The PickPen® technology reduces time of mRNA isolation from several hours to 10 minutes without loss of substantial amounts of mRNA. Even single live cell analysis can be performed in a reliable and reproducible way. Using conventional PCR, the theoretical limit of detection is one copy of a single-stranded DNA molecule. Efficient harvesting, quick mRNA isolation, and an optimized RT-PCR protocol allow reliable single-cell PCR.

Even stem cells in different development stages can be catapulted and cloned using the PALM® MicroBeam for subsequent expression profiling. Transfected cells after microinjection of drugs or genetic material can be used as source to investigate gene expression to check the effect of the applied substance.

We showed a proof-of-principle method by analyzing human PBGD gene. This method can be extended and then allows analysis and identification of specific genes which e.g. are involved in physiological processes in a complex of variable cell phenotypes.