Enhanced molecular analyses by combination of the HOPE-technique and laser microdissection

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Abstract

As part of an investigation aimed at illuminating the possibilities and limits of the HOPE-fixation and paraffin-embedding technique we here describe a novel procedure which was developed in order to combine the benefits of the HOPE-technique with the capabilities of laser microdissection. The presented procedure avoids the need for amplification of template-RNA thus facilitating reliable and reproducible results. The excellent preservation of nucleic acids, proteins, and morphology in HOPE-fixed, paraffin-embedded tissues enhances the molecular applications available to date with materials acquired by laser microdissection when compared to formalin fixed, paraffin-embedded tissues, thus substantially extending the methodological panel in tissue based research.

Introduction

Laser microdissection is a valuable tool for analysis of molecular parameters in purified cell populations or even single cells taken out of their native environment within tissues. This technology requires both an acceptable preservation of morphological details which is needed for exact dissection and the preservation of the nucleic acids which are analyzed. Fixation of tissues with formalin results in well preserved morphology but – to a high degree – leads to degradation of nucleic acids which substantially constricts the spectrum of applicable molecular techniques [4]. The novel HOPE-technique with subsequent paraffin-embedding, as an alternative to formalin, has been shown to result in a morphological preservation comparable to formalin-fixed, paraffin-embedded specimens [6]. Moreover, we described procedures which permit successful application of all common molecular techniques such as in situ hybridization [1,4,7], immunohistochemistry without antigen retrieval and for formalin-refractory antigens [1,3], PCR [8,11], RT-PCR [6,11], Western blot [9], Northern blot, and transcription microarrays [2] to HOPE-fixed, paraffin-embedded tissues. HOPE-fixed tissues can be used for preparation of tissue microarrays for enhanced high throughput analyses on the molecular level [5,12]. Using the HOPE-technique as its crucial methodological base, ex vivo model systems could be established, e.g. for the simulation of early events in human infections and detection of chemotherapy resistances in human cancer [7,13]. In addition to tissues, cell-culture preparations have been prepared.

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utilizing the HOPE-technique, which were then success-
fully applied to in situ hybridization targeting mRNA or
immunocytochemistry with excellent preservation of
morphological details [10]. In this study we describe the
use of HOPE-fixed, paraffin-embedded tissues for laser
microdissection and subsequent molecular analysis of
RNA-transcripts by real time RT-PCR. Results are set in
relation to those obtained with formalin fixed, paraffin-
embedded tissues from the same lesions. This real time
RT-PCR was unambiguously performed without any
amplification of RNA or total cDNA, which eliminates the
need for such hardly controllable steps necessary in estab-
lished procedures with formalin fixed tissues.

Materials and methods
Sample preparation
The specimens used were tumor-bearing or tumor-free
materials (at least 5 cm away from the tumor front) from
lobectomy or pneumectomy because of lung cancer. For
means of comparison, tissue samples from the same
organs were conventionally formalin-fixed and paraffin-
embedded or treated according to the HOPE-technique.

Fixation of tissues by application of the HOPE-technique
was carried out like previously described (6), starting with
an incubation of fresh tissue specimens in an aqueous
protection-solution (containing a mixture of different
amino-acids) overnight at low temperatures of 0–4°C
(DCS, Hamburg, Germany). Incubation in acetone fol-
lowed at 0–4°C. Tissues were then directly embedded
with paraffin.

Tissue sections from HOPE- or formalin fixed and paraffin
embedded (FFPE) human lung cancer tissue samples were
prepared on membrane mounted slides (PALM Mem-
braneSlides, P.A.L.M., Bernried, Germany).

HOPE-sections were deparaffinized either with isopropan-
ol (2 × 10 min at 60°C) or like the normal FFPE-sections
with xylene washes (2 × 10 min at room temperature).

All sections were stained with cresyl violet (1% w/v cresyl
violet acetate in 100% ethanol; Aldrich #86,098-0) for 1
min at room temperature, washed shortly in 70% and
100% ethanol and subsequently air dried.

Laser microdissection and pressure catapulting (LMPC)
LMPC was performed using a PALM MicroBeam system
with a pulsed UV-A nitrogen laser (337 nm). Using the
10× objective areas of interest were marked, cut and cata-
pulted into the cap of 0.5 ml microfuge tubes with adhe-
sive filling (PALM AdhesiveCaps, P.A.L.M., Bernried,
Germany). Smaller areas were pooled to reach average
sample sizes of 0.5–1 million square microns. After com-
pleted microdissection the remaining part of each tissue
section was cut out with a scalpel and collected in regular
0.5 ml microfuge tube (cut out section).

RNA extraction
Tissue samples were extracted using the RNeasy Micro Kit
(Qiagen, Hilden, Germany, #74004) following the manu-
facturer’s instruction manual. Briefly, the cells were dis-
solved in 350 μl of RLT lysis buffer, treated with DNasel
after the first washing step according to the manual and
finally the purified RNA was eluted in 12 μl of RNase free
water.

Spectrophotometry was performed for testing concentra-
tion and purity of RNA, which revealed well-preserved
RNA of high concentration throughout the samples.

Of each RNA sample 1 μl was tested in an Agilent Bioana-
lyzer 2100 using the RNA 6000 Pico LabChip kit (Agilent,
Waldbronn, Germany, #5065-4473) to determine the
RNA integrity.

Reverse transcription and RT-PCR
The synthesis of cDNA was performed using the 1st strand
cDNA synthesis kit for RT-PCR (AMV) (Roche, Penzberg,
Germany, #1483-188) following the manufacturer's
instructions. Briefly, 8.2 μl of RNA solution were reverse
transcribed in a final volume of 20 μl using random prim-
ers from the kit to ensure efficient transcription also of
fragmented or partially degraded RNA.

RT-PCR was performed using primers for the low abund-
ant reference gene human Hypoxanthine phosphoribo-
syltransferase (huHPRT, GenBank: M31642, pos. 383–
411 and pos. 613-591) producing a specific amplicon of
231 bp.

The PCR reaction was done in a LightCycler Instrument
using the Fast Start Master Plus SYBR Green system
(Roche, Penzberg, Germany) applying the following con-
ditions: 95°C for 10 min; 50 cycles at 95°C for 10 sec,
67°C for 10 sec, 72°C for 10 sec followed by a melting
curve analysis from 70° to 99°C in 0.1°C steps to control
specificity. The amount of RT-PCR product was automati-
cally assessed by real time fluorescence detection with the
LightCycler software package.

Results
HOPE-fixed, paraffin-embedded tissues displayed excel-
rent ‘formalin-like’ preservation of morphological details
after sectioning and cresyl violet staining with good adhe-
sion to the membrane mounted slides (PALM Mem-
braneSlides) used for laser microdissection (Fig. 1). Laser
microdissection was smoothly possible without any modi-
fication to the PALM MicroBeam system; the same holds
true for the pressure catapulting of the dissected material
Photomicrographs of HOPE-fixed tissues, deparaffinized and stained with cresyl violet (both 100× magnification). Sections were subjected to LMPC utilizing a PALM MicroBeam system. A: Pulmonary carcinoma with the area subjected for LMPC marked. B: Pulmonary carcinoma with the area subjected for LMPC microdissected and transferred to a microfuge tube by pressure catapulting.

**Figure 1**
Photomicrographs of HOPE-fixed tissues, deparaffinized and stained with cresyl violet (both 100× magnification). Sections were subjected to LMPC utilizing a PALM MicroBeam system. A: Pulmonary carcinoma with the area subjected for LMPC marked. B: Pulmonary carcinoma with the area subjected for LMPC microdissected and transferred to a microfuge tube by pressure catapulting.
into the collection tubes (Fig. 1). Extraction of RNA according to the protocol described above was achieved quickly. RNA-quality of the HOPE-fixed samples analyzed by using the Agilent Bioanalyzer was clearly superior to the formalin-fixed materials, which is exemplified in figure 2. In the real time RT-PCR (Fig. 3) similar results were obtained with deparaffinization utilizing xylene or isopropanol for the samples which have undergone LMPC and the cut out sections. Differences between cut out sections and LMPC can be explained by the differing amounts of starting material. FFPE materials – either LMPC or cut out sections – did not show any amplification of the targeted human Hypoxanthine phosphoribosyltransferase fragment. This is in good agreement with the results of RNA-quality analysis in FFPE materials obtained with the Bioanalyzer, which does not show any visualized RNA in these blocks (Fig. 2).

Discussion

Laser microdissection for analysis of molecular parameters in cell populations or single cells out of tissues requires acceptable preservation of morphology and nucleic acids. This cannot be achieved to a sufficient degree by the use of FFPE, which display degradation of nucleic acids if compared to fresh materials, which prompted us to this investigation of HOPE-fixed materials.

Figure 2
Results of RNA-integrity-testing by a Agilent Bioanalyzer 2100 using the RNA 6000 Pico LabChip kit. Lane 1 and 2 are HOPE-fixed materials and lane 3 is FFPE.

Figure 3
Real time RT-PCR targeting a 231 bp fragment of the RNA of human Hypoxanthine phosphoribosyltransferase. Green: HOPE, deparaffinized with xylene, cut out section. Black drawn through: HOPE, deparaffinized with isopropanol, cut out section. Black with dots: HOPE, isopropanol, whole slice before mounting on slide directly processed. Blue: HOPE, deparaffinized with isopropanol, LMPC. Red: HOPE, deparaffinized with xylene, LMPC. Black noncontinuous: two superposing samples, FFPE, both deparaffinized with xylene, cut out section and LMPC.
We showed that HOPE-fixed, paraffin-embedded tissues are well suited for laser microdissection. The well preserved morphology is comparable to formalin-fixed specimens and superior when compared to frozen sections. RNA of high quality can be extracted from the microdissected samples and subsequently being used for successful analysis by real time RT-PCR. These RT-PCR analyses can be performed without the need for any RNA amplification procedure. This results in higher specificity and reproducibility if compared to protocols which have to use such techniques due to degradation within the (usually formalin-fixed) specimens. Therefore results of real time RT-PCR with HOPE-fixed tissues are clearly superior to those obtained in formalin-fixed materials.

The combination of the enhanced molecular possibilities provided by the HOPE-technique with laser microdissection represents a novel tool for future tissue-based studies. Appropriate studies are underway to further extend these promising initial results.

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