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A High-Sensitivity, Medium-Density, and Target Amplification–Free Planar Waveguide Microarray System for Gene Expression Analysis of Formalin-Fixed and Paraffin-Embedded Tissue

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BACKGROUND: Many microarray platforms and their associated assay chemistries do not work properly with RNA extracted from formalin-fixed, paraffin-embedded (FFPE) tissue samples, a feature that severely hampers the use of microarrays in oncology applications, for which FFPE tissue is the routine specimen. Furthermore, the limited sensitivity of most microarray platforms requires time-consuming and costly amplification reactions of the target RNA, which negatively affects clinical laboratory work flow.

METHODS: We developed an approach for sensitively and reliably measuring mRNA abundances in FFPE tissue samples. This approach involves automated RNA extractions, direct hybridization of extracted RNA to immobilized capture probes, antibody-mediated labeling, and readout with an instrument applying the principle of planar waveguides (PWG). A 14-gene multiplex assay conducted with RNA isolated from 20 FFPE blocks was correlated to an analysis of the same with reverse-transcription quantitative real-time PCR (RT-qPCR).

RESULTS: The assay sensitivity for gene expression analysis obtained for the PWG microarray platform was <10 fmol/L, eliminating the need for target preamplification. We observed a correlation coefficient of 0.87 to state-of-the-art RT-qPCR technology with RNA isolated from FFPE tissue, despite a compressed dynamic range for the PWG system (a 2.9-log dynamic range for PWG in our test system vs 5.0 logs for RT-qPCR). The precision of the PWG platform was comparable to RT-qPCR (Pearson correlation coefficient of 0.9851 for PWG vs 0.9896 for RT-qPCR) for technical replicates.

CONCLUSIONS: The presented PWG platform demonstrated excellent sensitivity and precision and is especially well suited for any application for which fast, simple, and robust multiplex assays of RNA in FFPE tissue are required.

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Gene expression analysis is a powerful tool for the characterization of tissue or cells. With chip- or bead-based microarrays, whole-genome gene expression analysis has opened a multitude of new research areas, ranging from basic research to applications in personalized medicine. Numerous publications have described the identification of molecular markers for the characterization of cancers [e.g., breast cancer; reviewed in *(1)*]. The genetic signatures described in these publications suggest that 10–20 genes, but sometimes as many as 70, are required to obtain an informative subclassification of the cancer of interest *(2–7)*. Consequently, there is a need for a platform of low to medium throughput for detecting gene expression. Thus far, real-time PCR [or quantitative real-time PCR (qPCR)[3]] has been the accepted method in clinical settings. Although qPCR offers outstanding sensitivity, the 4 to 5 detection channels provided by current instruments limits multiplexing capabilities. Therefore, time-consuming and, in the case of manual pipetting, error-prone parallel measurements in multiple wells are needed. Furthermore, measures must be taken to minimize the high potential for contamination that affects amplification-based detection systems.

On the other hand, commercially available microarray platforms offer a high degree of multiplexing, but they are limited with respect to sensitivity and throughput *(8)*. Most importantly, their chemistries are usually not compatible with RNA extracted from formalin-fixed, paraffin-embedded (FFPE) tissue,

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[3] Nonstandard abbreviations: qPCR, quantitative real-time PCR; FFPE, formalin-fixed, paraffin-embedded; PWG, planar waveguide; RT-qPCR, reverse-transcription qPCR; Cq, quantification cycle.

which is fragmented and chemically modified. Consequently, such microarray platforms are not suited for most oncology applications, for which FFPE samples represent the commonly available material.

The system we describe overcomes the limitations of qPCR with regard to the level of multiplexing and those of conventional microarray platforms with regard to sensitivity and compatibility with FFPE tissue samples. On a high-sensitivity planar waveguide (PWG) microarray *(9)*, target RNAs are hybridized directly to DNA capture probes. The use of a fluorescently labeled antibody for recognizing and detecting RNA/DNA hybrids eliminates the need for any enzymatic amplification step. We use a panel of 14 genes and FFPE tumor material to compare this system with the qPCR approach. PWG microarrays exhibit a precision comparable to that of qPCR while simultaneously offering the potential for parallel measurements of approximately 100 gene expression markers from a single patient sample.

Materials and Methods

FFPE TISSUE SAMPLES

This study included 20 FFPE blocks of breast cancer tissue collected at 2 pathology laboratories in Germany: 15 blocks from HELIOS Clinics, Wuppertal (embedded in 2005) and 5 blocks from Charité, Berlin (embedded in 2003). The samples had been irreversibly anonymized; no clinical or patient-related information was available.

SAMPLE PREPARATION

Two consecutive 10-μm sections were cut from each patient block on a standard microtome (Reichert-Jung HN40; Leica Instruments), placed into individual 1.5-mL Sarstedt microtubes and stored at 4 °C for up to 3 months until extraction.

RNA EXTRACTION

The fully automated isolation of total RNA from FFPE tissue was performed on a Hamilton MICROLAB STARlet liquid-handling robot. Unless otherwise indicated, the robot, buffers, and chemicals are part of the VERSANT® kPCR Molecular System (Siemens Healthcare Diagnostics), which is commercially available in the EU but currently not available in the US. First, 150 μL FFPE buffer (BUFFER FFPE, a research reagent from Siemens Healthcare Diagnostics and not commercially available: 10 mmol/L Tris/HCl, pH 8.0, 0.1 mmol/L EDTA, and 20 g/L SDS, adjusted to pH 8.0) was added to each section and incubated for 30 min at 80 °C with shaking to melt the paraffin and start tissue lysis. After cooling, 50 μL of VERSANT 3.0 assay (bDNA) LYSIS REAGENT was added and incubated for 30 min at 65 °C. After lysis, residual tissue debris was removed from the lysis fluid with a 15-min incubation at 65 °C with 40 μL of silica-coated iron oxide beads. Beads with surface-bound tissue debris were separated on a magnet, and lysates were transferred to a standard 96-well plate with deep 2-mL wells, where total RNA and DNA was bound to 40 μL of fresh beads with shaking at room temperature. Chaotropic conditions were conferred by the presence of 600 μL lysis buffer (from the VERSANT kPCR Molecular System). Beads were magnetically separated, and the supernatants were discarded. Surface-bound nucleic acids were then washed 3 times, with magnetization, aspiration, and disposal of the supernatants. Nucleic acids were subsequently eluted by incubating the beads with 100 μL elution buffer (from the VERSANT kPCR Molecular System) for 10 min at 70 °C with shaking. Finally, the beads were separated, and the eluate was incubated with 12 μL of DNase I mix [2 μL DNase I (RNase free) and 10 μL 10× DNase I Buffer; Ambion/Applied Biosystems] to remove contaminating DNA. After incubation for 30 min at 37 °C, we combined the reaction mixtures containing DNA-free total RNA for 2 adjacent tissue sections and stored the combined mixture at −80 °C.

GENE EXPRESSION ANALYSIS WITH qPCR

The expression of 14 genes was assessed by a 1-step reverse-transcription qPCR (RT-qPCR). SuperScript® III Platinum® One-Step Quantitative RT-PCR System with ROX (Invitrogen) was used according to the manufacturer's instructions. Experiments were performed on an ABI PRISM® 7900HT Sequence Detection System (Applied Biosystems) under the following conditions: 30 min at 50 °C, 2 min at 95 °C, and 40 cycles of 15 s at 95 °C and 30 s at 60 °C. Relative expression values for the genes of interest were calculated as: $\Delta Cq = 20 - (Cq_{GOI} - Cq_{reference\ gene})$, where Cq is the quantification cycle and GOI is the gene of interest. *RPL37A*[4] (ribosomal protein L37a) was used as a reference gene. ΔCq values positively correlate with relative gene expression.

[4] Genes: *RPL37A*: ribosomal protein L37a; *CALM2*, calmodulin 2 (phosphorylase kinase, delta); *CHPT1*, choline phosphotransferase 1; *CXCL13*, chemokine (C-X-C motif) ligand 13; *ERBB2*, v-erbb2 erythroblastic leukemia viral oncogene homolog 2, neuro/glioblastoma derived oncogene homolog (avian) (aliases: *HER2*, *HER-2*); *ESR1*, estrogen receptor 1; *IGKC*, immunoglobulin kappa constant; *MLPH*, melanophilin; *MMP1*, matrix metallopeptidase 1 (interstitial collagenase); *OAZ1*, ornithine decarboxylase antizyme 1; *PGR*, progesterone receptor; *RACGAP1*, Rac GTPase activating protein 1; *TOP2A*, topoisomerase (DNA) II alpha 170kDa; *UBE2C*, ubiquitin-conjugating enzyme E2C; *XCP2*, xylem cysteine peptidase 2 (*Arabidopsis thaliana*); *pheB*, phenylalanine biosynthesis associated protein (*Bacillus subtilis*); *lysA*, diaminopimelate decarboxylase (*Bacillus subtilis*).

Table 1. Work flow for the PWG platform.[a]

Step	Time	Actions
Chip preparation	0.5 h	Assembly into carrier, equilibration at 42 °C in buffer PHB
Denaturation	5 min	Denaturation of hybridization mix
Hybridization	4–16 h	Replacement of buffer PHB by hybridization mix, incubation at 42 °C with shaking
Blocking	0.5 h	Replacement of hybridization mix by blocking solution, incubation at room temperature with shaking
RDAb[b] incubation	1 h	Replacement of blocking solution by antibody dilution, incubation at room temperature with shaking
Preparation for readout	1 min/array	Replacement of antibody dilution by buffer SB
Readout	2.5 min/array	Detection in PWG instrument
Total time for 24 samples	7–18 h	

[a] Listed are the individual steps and time requirements for the work flow on the PWG platform. The ZeptoCARRIER can accommodate 24 arrays in a single run. Also given is the total time for 24 samples, which depends on the hybridization time.
[b] RDAb, RNA/DNA hybrid–specific antibody.

Human genomic DNA (Roche Diagnostics) was used as a positive control for qPCR. All PCR assays were performed in triplicate. The qPCR primers and hydrolysis probes for the genes *CALM2* [calmodulin 2 (phosphorylase kinase, delta)], *CHPT1* (choline phosphotransferase 1), *CXCL13* [chemokine (C-X-C motif) ligand 13], *ERBB2* [v-erbb2 erythroblastic leukemia viral oncogene homolog 2, neuro/glioblastoma derived oncogene homolog (avian)], *ESR1* (estrogen receptor 1), *IGKC* (immunoglobulin kappa constant), *MLPH* (melanophilin), *MMP1* [matrix metallopeptidase 1 (interstitial collagenase)], *OAZ1* (ornithine decarboxylase antizyme 1), *PGR* (progesterone receptor), *RACGAP1* (Rac GTPase activating protein 1), *RPL37A*, *TOP2A* [topoisomerase (DNA) II alpha 170kDa], and *UBE2C* (ubiquitin-conjugating enzyme E2C) were identified with Primer Express™ software (Applied Biosystems) according to the manufacturer's instructions. Primer and probe sequences are documented in Table 1 in the Data Supplement that accompanies the online version of this article at http://www.clinchem.org/content/vol55/issue11.

IN VITRO TRANSCRIPTION OF SPIKE-IN RNAS

In vitro transcription of linearized pBluescript KS+ plasmids (Stratagene/Agilent Technologies) containing the coding sequences of *Bacillus subtilis* control genes *lysA* (diaminopimelate decarboxylase) and *pheB* (phenylalanine biosynthesis associated protein) or the *Arabidopsis thaliana* control gene *XCP2* (xylem cysteine peptidase 2) were performed with the T7 MEGAscript High Yield Transcription Kit (Ambion/Applied Biosystems) according to the manufacturer's instructions. The concentration of the resulting antisense RNA was measured spectrophotometrically on a NanoDrop 1000 (Thermo Scientific); the final concentration was adjusted to 50 ng/μL with water, dispensed into aliquots, and stored at −80 °C.

PREPARATION OF PWG MICROARRAYS

PWG substrates were supplied by Oerlikon and subsequently coated with either an epoxysilane polymer (Nexterion E; Schott) or a polyvinylamine polymer (Lupamin 9095; BASF). Lupamin coating and spotting of both Nexterion and Lupamin chips was performed by Zeptosens, a division of Bayer (Schweiz). Spotted chips could be stored for several months at 4 °C without a noticeable increase in the background fluorescence (data not shown). For this reason, the manufacturing of PWG microarrays was not included in the work flow analysis presented in Table 1.

Each PWG chip carried 6 identically spotted arrays. The spotting area of each array was 5 × 6.1 mm, which was covered with 208 spots with an approximate diameter of 120 μm and a center-to-center distance of 380 μm. For the measurement of each transcript, we aligned quadruplicate spots in a row parallel to the grating. Therefore, a total of 52 transcripts in 13 columns and 16 rows could be analyzed on the PWG chips used in this study.

DNA capture probes for PWG chips were between 53 and 71 nucleotides long and were purified by PAGE or HPLC. The probes were designed in the antisense direction to the target genes and in the sense direction to the bacterial and plant control genes.

The sequences of the PWG capture probes and details regarding their design are provided in Table 2 in the online Data Supplement.

HYBRIDIZATION AND DETECTION

Each RNA isolated from FFPE tissue was measured in replicates, namely on 2 arrays located on the same chip. The PWG instrument (ZeptoREADER, including the ZeptoCARRIER and SensiChip View 2.1 software) and the corresponding buffers (PHB, 2× HB, and SB) were purchased from Zeptosens. PWG chips were assembled into the ZeptoCARRIER and incubated with 200 μL PHB buffer in a heating/shaking device (ThermoTwister; Quantifoil Instruments) for 30 min at 42 °C and 400 rpm. We heated 100 μL of hybridization mix [40 μL of the RNA eluate, 25 μL 4× HB hybridization buffer (based on the 2× HB buffer and produced in collaboration with Zeptosens), 300 mL/L formamide, and 2 g/L SDS] for 5 min at 95 °C and then quenched the reaction on ice. The PHB buffer was replaced by the hybridization mix, and the chips were incubated at 42 °C with shaking, either for 4 h (Lupamin-coated chips) or overnight (Nexterion E–coated chips).

RNA/DNA hybrids were detected with an Alexa 647–conjugated RNA/DNA hybrid–specific antibody *(10)*.

After the incubation, the hybridization mix was replaced with 200 μL blocking solution [25 g/L skim milk powder (Fluka/Sigma–Aldrich Chemie) and 1 mL/L Tween 20 in PBS (Phosphate-Buffered Saline (PBS) 7.4 (1X) liquid); Invitrogen] and incubated at 25 °C for 30 min. The blocking solution was then replaced with RNA/DNA hybrid–specific antibody solution (final concentration, 1.2 mg/L) diluted into 25 g/L dextran sulfate, 15 g/L BSA, and 1 mL/L Tween 20) and incubated with shaking for 1 h at 25 °C. The antibody solution was then replaced with 200 μL SB buffer.

Measurements were made with a ZeptoREADER PWG instrument according to the manufacturer's instructions. Four different exposure times (1, 3, 10, and 30 s) were used. SensiChip View 2.1 software was used to analyze TIFF images at a fixed spot diameter of 125 μm, with "center of gravity" as the spot-alignment method and an interzone of 13 pixels. The signal intensity of each spot was calculated as the median of the pixel intensities. Owing to the array design, we used the mean intensity of the quadruplicate spots. All arrays were normalized to the mean signal intensity of the housekeeping gene *CALM2*.

STATISTICAL EVALUATION

For statistical analyses, we used Prism® 4 (GraphPad Software) and Microsoft Excel 2003 software. We calculated arithmetic means, medians, and CVs and calculated the Pearson correlation coefficient for correlation analyses. To compare signal intensities derived from the PWG instrument with qPCR-derived Cq values, we converted the former to logarithms.

Results

CHIP LAYOUT AND WORK FLOW

Because of the characteristics of the PWG excitation and detection principle of an evanescent field, both the amplification of the target nucleic acid and the steps of array washing were obsolete. In addition, applying a detection system based on an RNA/DNA hybrid–specific antibody permitted the use of RNA isolated from FFPE material without any further manipulation, such as conversion into cDNA or cRNA. The RNA had only to be denatured to be capable of hybridizing to the DNA capture probes. This approach produced a fairly short work flow with few manual interventions (Table 1). Depending on the chip coating, the hybridization time could be shortened from the generally applied overnight incubation to a 4-h incubation with only a small decrease in performance (data not shown).

PRECISION

The SensiChip View software reported both the median signal intensity of the pixels of each spot and the signal-to-noise ratio for each spot calculated from a local background value. The data were normalized to the *CALM2* housekeeping gene. The signal-to-noise ratio was set to at least 3 for the gene to be called as present. Fig. 1, A and B, shows the median intensities of the quadruplicate spots for 2 typical tumor blocks, and the mean intensities of duplicate arrays on the same blocks (Fig. 1, C and D) were plotted in order of increasing signal intensity. Only valid signals (i.e., with a signal-to-noise ratio $\geq$3) were plotted. The mean CV for the quadruplicate spots over all analyzed arrays (intraarray variation) was 10%, and the interarray CV for duplicate arrays was 16% after normalization.

SENSITIVITY

To test the sensitivity of the system, we used RNA transcribed in vitro from bacterial (*Bacillus subtilis*) and plant (*Arabidopsis thaliana*) genes in titration experiments. We tested different RNA mixtures at 4 concentrations (0.001–1 pmol/L). We plotted all signal-to-noise ratios regardless of validity (Fig. 2A), as well as the mean intensities of signals with signal-to-noise ratios $>$3 (Fig. 2B). Each point in the plots represents the mean of replicate arrays. For this plot, we used signals with the longest exposure time (30 s) to obtain the best sensitivity. This approach led to a saturated signal for the highest concentration (1 pmol/L). Although the SensiChip View software provides a "dynamic range extension" option, in which signals from 2 different exposure times can be combined mathematically, we did not apply this feature in this case. The titration experiment yielded a limit of detection for all 3 analyzed genes of $>$0.01 pmol/L (i.e., 1×10^{-14} mol/L).

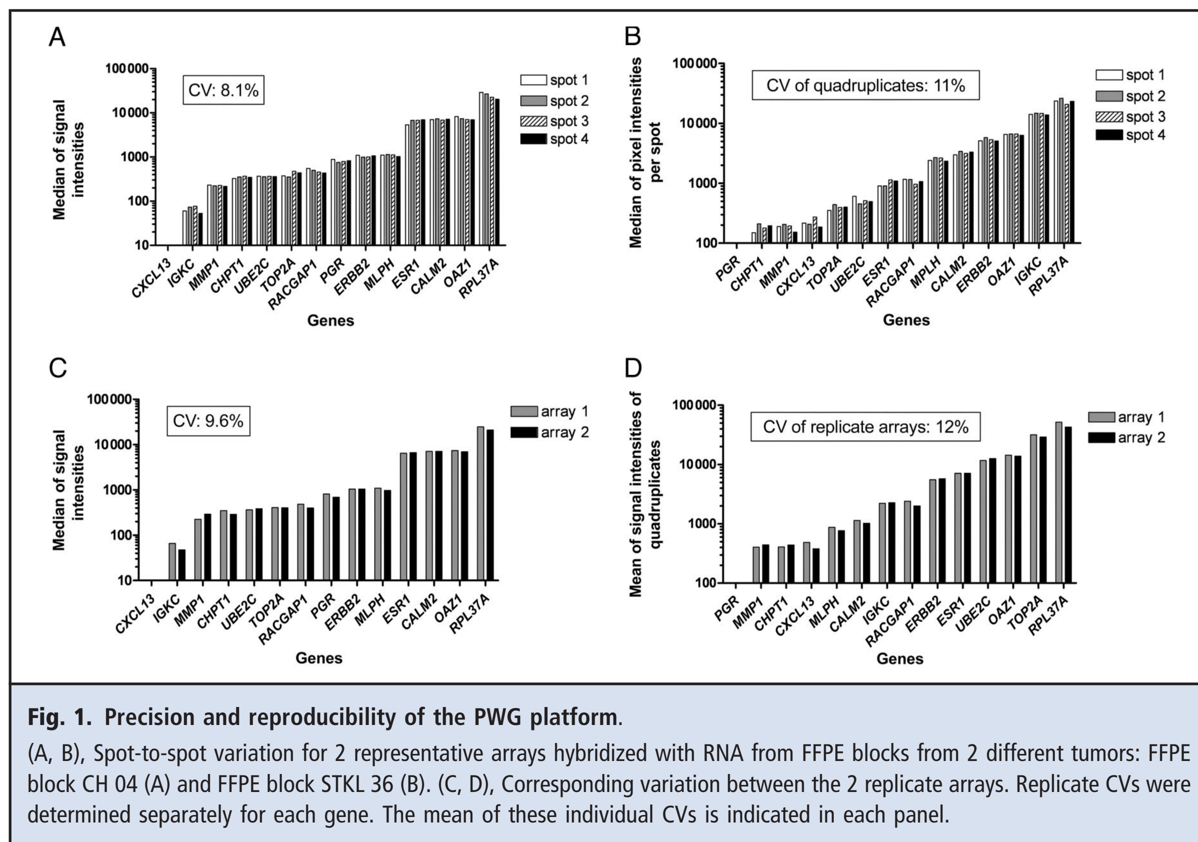

Fig. 1. Precision and reproducibility of the PWG platform.

(A, B), Spot-to-spot variation for 2 representative arrays hybridized with RNA from FFPE blocks from 2 different tumors: FFPE block CH 04 (A) and FFPE block STKL 36 (B). (C, D), Corresponding variation between the 2 replicate arrays. Replicate CVs were determined separately for each gene. The mean of these individual CVs is indicated in each panel.

CORRELATION TO RT-qPCR ASSAYS

The technique widely accepted for gene expression analysis of low to medium throughput is RT-qPCR. We used both RT-qPCR and the PWG platform to analyze 14 human genes (11 relevant to breast cancer and 3 housekeeping genes) in pooled total RNA isolated from 2 sections each of 20 FFPE tumor blocks. All genes were detectable with the RT-qPCR method, whereas some genes were undetectable with the PWG platform. In particular, *CXCL13* and *PGR* were below the limit of detection on the PWG platform in 10 and 7 FFPE samples, respectively (Table 2). The 2 methods performed similarly well with respect to reproducibility (Fig. 3). The mean SD between replicates for RT-qPCR and the PWG platform was 0.19 Cq and 0.23 $\log_2$ (signal intensities), respectively (PWG platform, replicate arrays; RT-qPCR, triplicate measurements; data not shown), and the mean correlation coefficient between replicates was 0.9896 and 0.9851 for RT-qPCR and the PWG platform, respectively (Fig. 3C). Of note is that the replicates were measured on the same plate in the RT-qPCR analysis. The plate-to-plate variation would add additional noise to these results.

The RT-qPCR assay is known to have a significantly higher dynamic range than array-based methods. This observation was confirmed in the current study. The dynamic ranges of the RT-qPCR and PWG analyses were 5.0 logs and 2.9 logs, respectively (data not shown). Nevertheless, a good mean correlation coefficient over all samples of 0.8833 (Fig. 4) showed that both methods were similarly well suited to analyze gene expression in these samples.

Discussion

Previous evaluations of microarray technologies have produced conflicting results with respect to both their reproducibility and their interplatform comparability *(11)*. In a series of landmark publications, however, the MicroArray Quality Control (MAQC) consortium proved that the currently marketed gene expression platforms are reliable with regard to intra- and interplatform reproducibility *(12)*. Still, gene expression microarrays have not yet made their way to routine diagnostic applications. One of the reasons for this fact is the limited sensitivity of such platforms, which usually demand time-consuming amplification reactions of the often limited patient sample material.

We have presented a medium-density array system based on PWG technology that is capable of performing high-sensitivity gene expression analysis. The arrays used in this study allow measurements of 52

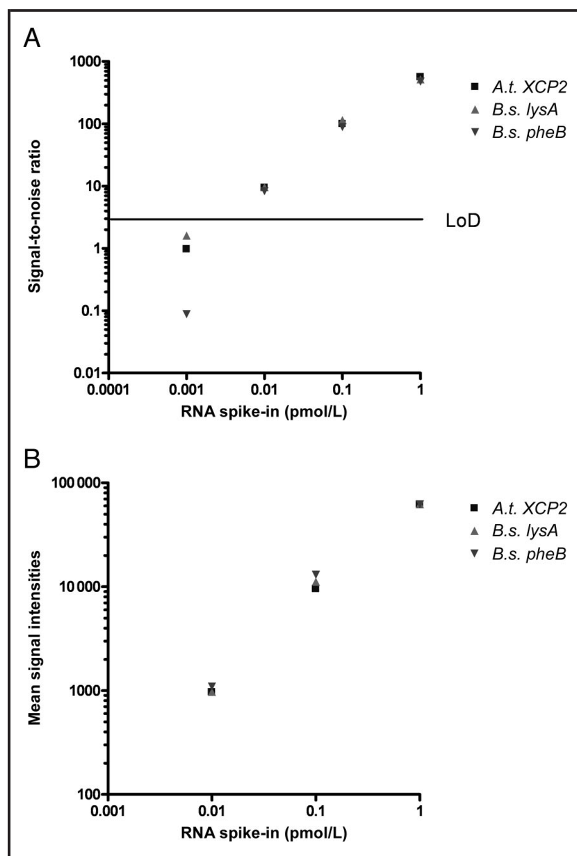

Fig. 2. Limit of detection (LoD) on the PWG platform. Spike-in experiments with RNA transcribed in vitro for control genes for exposure times of 30 s. (A), The signal-to-noise ratio is plotted, and the defined LoD value of 3 is indicated. (B), Corresponding signal intensities (mean of replicate measurements for signal-to-noise ratios >3). *A.t.*, *Arabidopsis thaliana*; *B.s.*, *Bacillus subtilis.*

Table 2. Number of FFPE samples for which the respective genes were detected.[a]

Gene	RT-qPCR	PWG
CHPT1	20	19
CXCL13	20	10
ERBB2	20	20
ESR1	20	18
IGKC	20	17
MLPH	20	16
MMP1	20	17
PGR	20	13
RACGAP1	20	17
TOP2A	20	20
UBE2C	20	19
CALM2	20	20
OAZ1	20	20
RPL37A	20	20

[a] Given is the number of FFPE samples (of 20 total) for which the respective genes could be detected with the 2 methods.

transcripts in quadruplicate measurements. As is shown in Fig. 1, the very high reproducibility of quadruplicate measurements (replicate CVs of approximately 10%) therefore may allow a reduced number of replicates on the chip. This reduction, together with a slight reduction in the spotting pitch size and/or an increase in the spotting area, could easily allow up to 100 transcripts to be analyzed on this chip. The PWG detection method of a surface-confined fluorescence excitation by an evanescent wave in PWG arrays leads to a substantial reduction in the background level and a 100-fold increase in the signal-to-noise ratio, compared with the widely distributed array detection system of conventional confocal microscopy *(9)*. This design increases the sensitivity, thereby permitting the omission of the target amplification required for most platforms of gene expression detection. In combination with a fluorescently labeled antibody specific for RNA/DNA hybrids, target RNA could be hybridized directly to the capture probes immobilized on the chip. Consequently, this approach does not require any enzymatic step, and it allows excellent compatibility with the usually heavily fragmented and modified RNA in FFPE material. The system is also compatible with RNA isolated from fresh tissue or cell suspensions. In such cases, however, a preceding step of chemical or physical fragmentation of the RNA is recommended, because it facilitates the formation of RNA/DNA hybrids on the chip (data not shown).

The aforementioned features of the labeling approach we have presented are obviously advantageous with respect to cost and work flow, given that conventional labeling procedures that often include reverse-transcription and/or in vitro transcription reactions are time-consuming and costly. Another beneficial aspect of the PWG principle is its ability to detect specific signals in the presence of unbound fluorescence signal. Washing steps are therefore obsolete, thus simplifying and shortening the work flow substantially. We were also able to reduce the hybridization time for a particular chip coating (Lupamin coating) to as little as 4 h. This reduction enabled us to run gene expression experiments within a single working shift. We have not optimized the hybridization conditions accordingly for the chip coating used for the RT-qPCR correlation study and therefore used an overnight hybridization incubation.

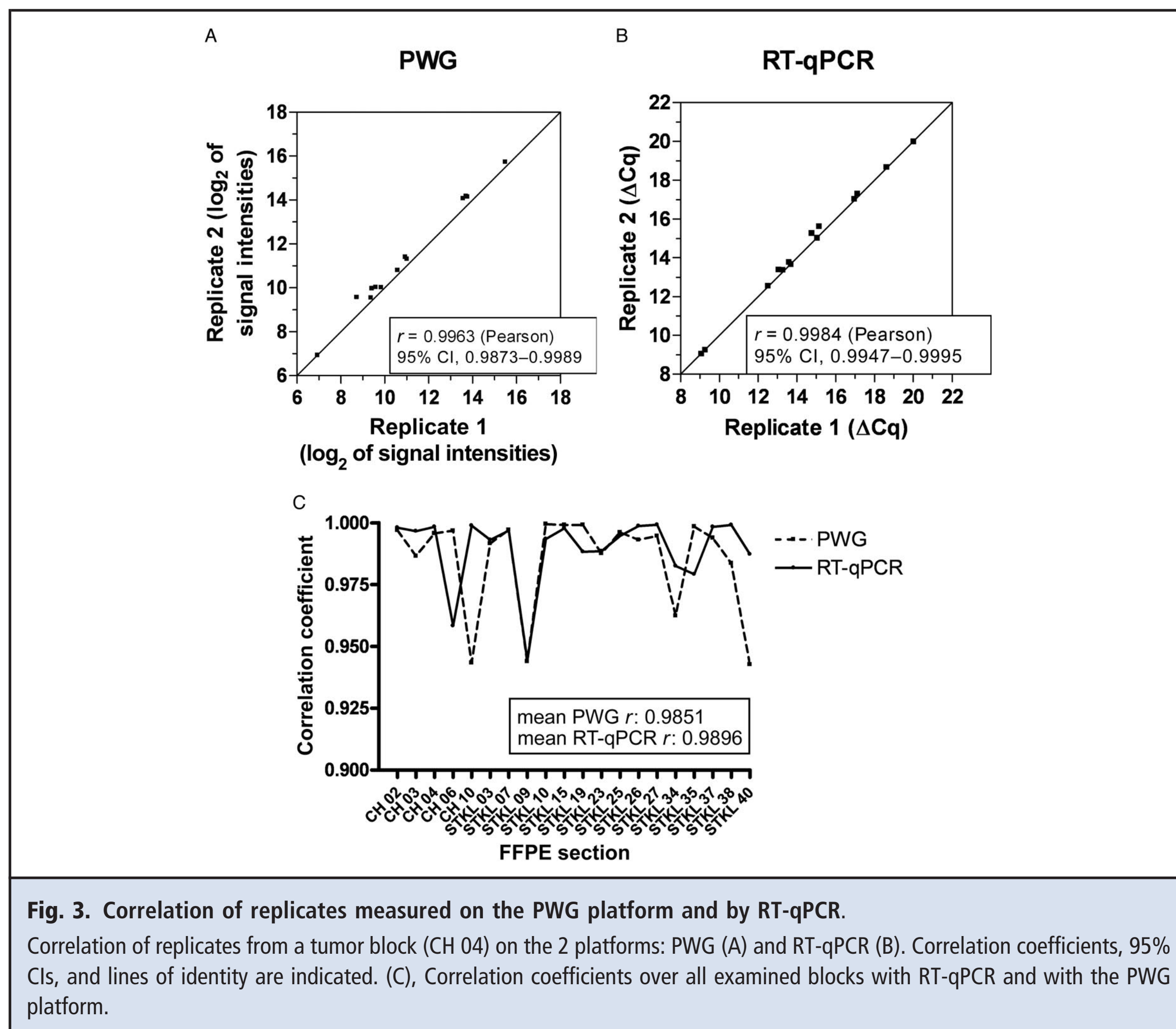

Fig. 3. Correlation of replicates measured on the PWG platform and by RT-qPCR.
Correlation of replicates from a tumor block (CH 04) on the 2 platforms: PWG (A) and RT-qPCR (B). Correlation coefficients, 95% CIs, and lines of identity are indicated. (C), Correlation coefficients over all examined blocks with RT-qPCR and with the PWG platform.

The sensitivity of the PWG platform was measured by spike-in titration experiments with RNA transcribed in vitro. The limit of detection was >10 fmol/L (i.e., 1×10^{-14} mol/L), which is superior to other array-based platforms, for which target amplification is mandatory. RT-qPCR is known to be very sensitive and capable of outperforming most other systems in this respect. This is also true for the analysis performed in this study. The maximum number of transcripts measured was 280 (14 genes in 20 FFPE samples). All 280 transcripts were detected with RT-qPCR, whereas 34 of 280 transcripts were below the limit of detection of the PWG platform. Although there is no doubt that the sensitivity of RT-qPCR is greater than the sensitivity of the PWG approach, greater sensitivity does not always add valuable information with respect to a subsequent clinical decision. For example, the expression of *ESR1* corresponds to either an estrogen receptor–positive or an estrogen receptor–negative status, and the cutpoint for this decision is approximately a ΔCq value of 14 in this experimental setting *(13)*. The additional distinction between low expression and complete absence of the gene (i.e., a ΔCq value <14) does not increase the information value of the result. Nevertheless, greater sensitivity might provide valuable information for other genes.

In the present study, RNA was isolated from FFPE tissue, analyzed for the expression of a panel of genes with PWG chips, and compared with analysis by RT-qPCR. Both methods had good precision and were comparable with each other (Fig. 3). Worth mentioning is that the replicates for the RT-qPCR method were measured on the same plate; a slightly higher imprecision would be expected if plate-to-plate variability were added. The results obtained with the 2 methods are well correlated. Plotting all of the data together yields a correlation coefficient of 0.8833 (Fig. 4C). Given that the 2 methods use different amplification

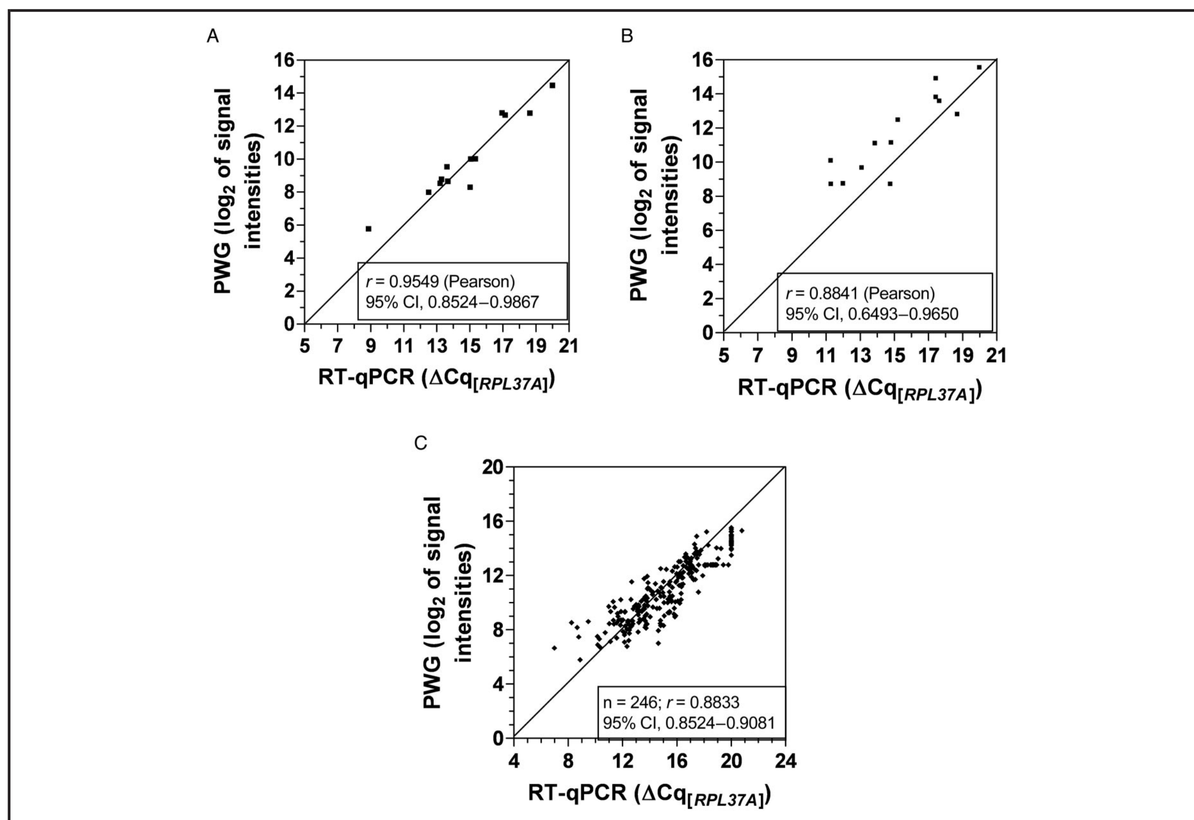

Fig. 4. Analyses of RNA production on the PWG platform and in the RT-qPCR assay.

(A, B), Correlation between gene expression results for detectable genes measured with the PWG platform and with RT-qPCR technology. Normalized data are shown for block CH 04 (*CXCL13* undetected by PWG) (A) and block STKL 36 (*PGR* undetected by PWG) (B). (C), Correlation of all 20 tumor blocks. Pearson correlation coefficients, 95% CIs, and lines of identity are indicated.

and detection principles, this correlation appears very convincing.

In summary, we present a PWG-based gene expression platform that allows reliable analysis of gene expression in a medium-throughput application and has the potential for the analysis of up to 100 genes in parallel within a single workday. The system performs well with RNA isolated from FFPE samples without requiring target amplification and hence without the risk of introducing an amplification bias. Importantly, the system maintains a very good correlation with the current state-of-the-art technology, RT-qPCR.

Author Contributions: *All authors confirmed they have contributed to the intellectual content of this paper and have met the following 3 requirements: (a) significant contributions to the conception and design, acquisition of data, or analysis and interpretation of data; (b) drafting or revising the article for intellectual content; and (c) final approval of the published article.*

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