2D-PCR: a method of mapping DNA in tissue sections†

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A novel approach was developed for mapping the location of target DNA in tissue sections. The method combines a high-density, multi-well plate with an innovative single-tube procedure to directly extract, amplify, and detect the DNA in parallel while maintaining the two-dimensional (2D) architecture of the tissue. A 2D map of the gene glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was created from a tissue section and shown to correlate with the spatial area of the sample. It is anticipated that this approach may be easily adapted to assess the status of multiple genes within tissue sections, yielding a molecular map that directly correlates with the histology of the sample. This will provide investigators with a new tool to interrogate the molecular heterogeneity of tissue specimens.

Introduction

Variations in DNA status (mutation, epigenetic modification, insertions, deletions) by location within a tissue section are of interest for elucidating their roles in biology and as clinical markers for diseases. However, to study the molecular basis of normal and pathological conditions, many researchers identify genes of interest from tissue specimens by examining cell homogenates (all the cells in the sample mixed together). Since tissue comprises a complex milieu of diverse cell populations that define the local microenvironments, combining the multiple cell populations into one measurement masks a tremendous amount of information. Therefore, to achieve a more complete view, DNA variability within a tissue sample needs to be correlated with the type of cell from which it originates (for example, stroma *vs.* epithelium), the physical location within the tissue section, and/or histopathological features (for example, a specific disease lesion). This is highlighted by the interest in techniques such as *in situ* polymerase chain reaction (PCR), and more recently by the increasing use of laser capture microdissection (LCM).

The majority of biological studies that extract, purify, and amplify genes or gene products from homogenized tissue to enable their detection utilize methods such as PCR,[1] reverse transcriptase (RT)-PCR,[2] quantitative PCR (qPCR),[3] qRT-PCR,[4] nano-litre qPCR,[5] digital RT-PCR,[6] DNA microarrays,[7,8] SAGE,[9] oligonucleotide microchips,[10] and OpenArrays.[11] These techniques are capable of impressive sensitivity and high-throughput capacity, while also being reliable and consistent among different laboratories. However, due to the homogenization step, they do not preserve the original 2D histological correlation between the locations of different cell types and their underlying molecular information.

Microdissection methods, including UV cutting,[12] LCM,[13] and its variants expression microdissection (xMD)[14] and immmuno-LCM,[15] were created specifically to work with histological tissue sections containing particular cell populations rather than homogenates. These approaches preserve the histological information and can be used in conjunction with sensitive and reliable molecular biology methods, such as PCR, for the molecular analysis of the tissue. For example, LCM has been used to isolate various cell populations of human prostate tissue to identify regional epigenetic alterations associated with the tumor microenvironment.[16] LCM allows an operator to isolate selected clusters of particular cell populations under microscopic visualization. A large number of like clusters are placed together into a standard 0.5 mL microcentrifuge tube and then studied with the same protocols used on tissue homogenates. As a result of the information on cellular location, as well as its reliability, LCM is now a widely used technique. However, creating a complete molecular profile across an entire tissue section is prohibitively time-consuming with the current microdissection techniques.

There are techniques for *in situ* mapping of genes throughout a tissue section, including catalyzed reporter deposition,[17] *in situ* PCR,[18,19] rolling circle amplification,[20] or branched DNA.[21,22] These techniques aim to obtain the molecular profiles of the cells in their original milieu, preserving the correlation with histological and immunohistochemical[23] examination. While this works well for some samples and targets, a widely accepted platform for 2D spatial molecular analysis has not yet emerged due to issues such as background autofluorescence[24] and the need to re-optimize on a case-by-case basis.[24] To overcome these

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† Electronic supplementary information (ESI) available: A figure showing the pressure compression rig, the electrophoresis gel validations of the 2D-PCR method, and complete details of the miniaturization studies, including the microfabrication, reagent loading by capillary action, cross-talk studies, demonstration of PCR, and discussion. See DOI: 10.1039/b910807f

issues, it is typical to either amplify the target genes[18–20] or the reporter molecules binding to the target genes[17,21,22,24] prior to visualization, and a few studies have even attempted multiple rounds of signal amplification[25,26] to improve the signal-to-noise ratio. Nevertheless, the *in situ* techniques have not seen the same level of acceptance as the well-established techniques for visualizing proteins.[23]

In this paper, we present the first lab-on-a-chip method to visualize target DNA across a tissue section. This will allow, upon further development, the mapping of alterations in genes, such as loss of heterozygosity (LOH), single nucleotide polymorphism (SNP), changes in copy number, and methylation, and correlating these changes with the tissue histology. This novel approach combines the strengths of three existing methods: the amplification power of PCR, the ability to examine clusters of cells using LCM, and the ability to localize molecular targets across a tissue section by *in situ* methods. Our approach, which we call 2D-PCR, makes use of a miniature array of wells together with protocols that allow tissue lysis, DNA amplification, and target detection within the same well. Proof-of-concept for this method was demonstrated *via* the creation of a 2D detection map, showing the presence/absence of the target DNA, on the millimetre scale, but resolution can be further improved to the micro-scale based on our preliminary findings. In principle, this approach can be miniaturized to the level of a few cells per well, the same resolution as achieved with LCM but with more comprehensive study of the tissue section, thereby advancing studies of gene expression as a function of the type of cell and its position within a tissue. We have also established this method of mapping DNA as a step towards the more challenging task of mapping mRNA and microRNA.

DNA mapping methodology

The 2D-PCR concept is illustrated in Fig. 1. A tissue is sectioned and transferred into a multi-well array device. The DNA is then extracted from the tissue and subsequently amplified by PCR in the same wells. This method allows mapping of genes while maintaining the reliability and specificity of PCR for detection. The PCR products can be detected either within the wells or after products are transferred onto one or more detection membranes.[27]

The steps to create the 2D map of DNA in tissue are illustrated schematically in Fig. 2. The multi-well device consists of a plate

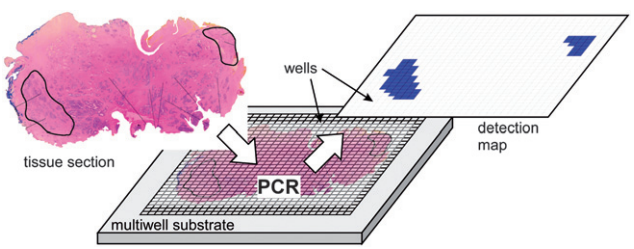

Fig. 1 2D-PCR is achieved by transferring a tissue section vertically into a multi-well array device, isolating tissue subregions. The DNA is extracted and then amplified by the polymerase chain reaction. A detection map is created by imaging the amplified DNA, for example using a DNA-intercalating dye.

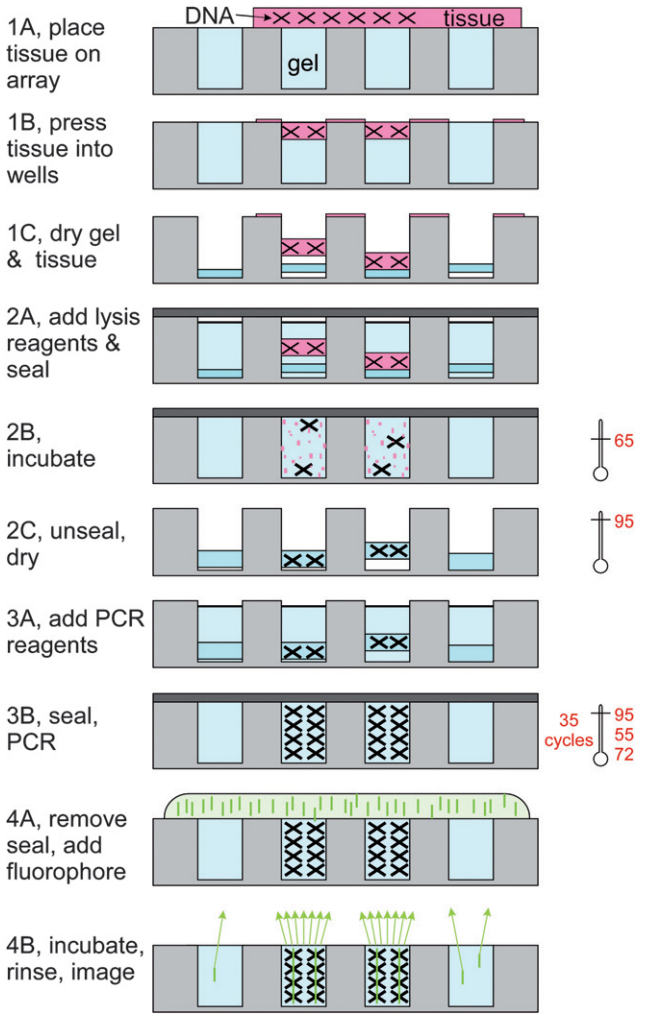

Fig. 2 Experimental protocol used to demonstrate the 2D-PCR concept. Tissue is transferred into wells, isolated, and lysed to extract the genomic DNA (represented by "×"s). PCR is performed to amplify the target DNA, and the targets are visualized with fluorescent dye (indicated by vertical lines "|").

with an array of through holes sealed on the bottom side to form the wells. The wells are pre-filled with agarose, which immobilizes the chemical species to prevent cross-contamination between neighboring wells. In step one, the tissue section is transferred onto the surface of the multi-well array (step 1A) and then pressed onto the surface (1B). The contents of the well are dehydrated to enable the addition of lysis reagents (1C). In step two, the tissue is lysed to release genomic DNA into solution. Lysis reagents are added to the wells, which are sealed (2A) and incubated at 65 °C (2B). Sealing prevents evaporation of reagents during the incubation, and heating activates the enzyme in the lysis reagent. The sealing film is removed, and the water in the wells is removed by dehydration at 95 °C (2C) to enable the subsequent addition of aqueous reagents. In step three, the DNA is amplified. PCR reagents are added (3A), and the device is re-sealed and heat cycled (3B). In step 4, the DNA is imaged. After unsealing the device, an intercalating fluorophore is added and allowed to incubate (4A). After rinsing off the dye solution, the fluorescence coming from the array is recorded and quantified (4B).

Materials and methods

Device fabrication

Devices were constructed from perforated aluminium (alloy 3003H14, Perforated Metals Plus) obtained in 30.5 × 30.5 cm sheets with 1.27 mm thickness. The through holes were used as wells/vials and had a 1.6 mm diameter and a 2.38 mm staggered center-to-center spacing. The sheets were cut into 3 × 3 cm pieces for the experiments and cleaned with 100% ethanol. These plates were used because they had the smallest commercially available hole size, near the size of a 1 mm pipette tip. They also had a flat surface and low aspect ratio holes to facilitate the transfer of tissue.

Low melting point (LMP) agarose was added into the through holes as an immobilizing medium because of its compatibility with a variety of enzymatic reactions,[28] including DNA polymerase and reverse transcriptases. LMP agarose (Lonza 50081) was mixed 2% by weight in 30 mL of water in a sterilized beaker and microwaved at 800 W for 60 s. After the solution cooled to 50 °C, the cleaned aluminium plates were dipped into the molten agarose solution for a few seconds to fill the wells by capillary action.

The back (burred) side of the plate was sealed with an 89 μm thick adhesive-backed fluoropolymer film (McMaster 5805T11) that was cut to 3 cm squares. The sealing film was rinsed with ethanol, air dried, and placed in contact with the multi-well device. The plate was then placed film-side-down on a flat surface and pressed firmly on all corners to ensure sealing. This sealing film remains permanently on the bottom side of the device. A seal of 51 μm thick Kapton film (McMaster 2271K72) was also ethanol cleaned and then added to the smooth top side of the plate. A large, flat, 2 pound weight was placed on top of the seal to create a smooth agarose surface for the tissue to be placed onto in the next step.

Tissue sectioning and transfer

Two types of tissue were used in these studies: frozen tissue samples from a human prostate with benign hyperplasia and breast tissue with lobular carcinoma. Both specimens were obtained at the National Institutes of Health (NIH) as part of an IRB-approved clinical protocol for molecular analysis. Patient identifiers were removed prior to the study. Tissue specimens were snap frozen and embedded in O.C.T. compound embedding medium (Sakura Fine-Tek, Torrance, CA). The tissue histology was evaluated by a pathologist (Rodriguez-Canales) to confirm the presence of viable cells in the tissue blocks. The blocks were then sectioned using a Leica CM1850UV cryostat. Prostate tissue sections measured approximately 40 mm × 20 mm with 8 μm thickness, and breast tumor tissue sections measured approximately 5 mm × 15 mm with 12 μm thickness.

For 2D gene mapping, the lobular breast carcinoma tissue block was stained so that it could be localized in the wells visually after transfer to compare against subsequent fluorescent detection of tissue DNA by 2D-PCR. The tissue block was thawed at room temperature and stained in an Eosin Y (0.05% wt/v) bath for 2 min. It was immersed in 70% ethanol for 5 min and 15% ethanol for 15 min, and then re-embedded in O.C.T. on dry ice for 10 min.

Tissue sections were transferred to the multi-well device immediately after sectioning while still frozen to faithfully preserve the 2D geometry. After removing the top surface Kapton sealing film, the device was placed top-side-down onto the tissue section. Since the device was at room temperature, the tissue section and O.C.T. compound melted locally, adhering the tissue to the device, in a similar way that cryostat sections are typically adhered to glass slides for histological evaluation. The tissue overlying each well was then pressed into the well by force: the tissue was covered with a fresh Kapton sealing film and the plate/tissue/film was compressed at 150 psi (pounds per square inch) and heated to 95 °C for 5 min. The device was then flipped and dehydrated as described below, so that the agarose would dry on top of the tissue to immobilize the DNA and thereby prevent cross-contamination between wells.

Device heating and sealing

To heat the wells for tissue transfer, cell lysis, and PCR while simultaneously preventing evaporation and cross-contamination of the wells due to internal vapor pressure, it was necessary to seal the wells of the device. A standardized procedure was developed to repeatedly remove and re-apply these seals. Wells were reversibly sealed with a fresh 51 μm thick Kapton film during each step of the procedure. This sealing film was rinsed with ethanol, air dried, and placed in contact with the multi-well device. The film and multi-well plate were compressed with 150 psi of force and heated using a customized compression–heating rig. The rig consisted of a steel frame supporting a weight scale, a PCR thermocycler with a flat heating surface, and a screw-type compression plate (see the ESI† for a figure).

The multi-well plate device was placed on the flat surface of the thermocycler (Alpha Unit Flat Block/PTC-200 DNA Engine, BioRad) and 200 μL of mineral oil were applied to the heater surface to improve thermal conductivity. The device was covered with a 1″ thick Plexiglass block for thermal insulation and a 1″ thick aluminium block for force distribution.

After the heating procedure, the contents of the wells were solidified by cooling to 0 °C for 10 min. Solidifying the agarose, which entrapped the DNA and reagents, ensured that the contents from one well did not move into any adjacent wells and that no fluid adhered to the sealing film upon removing the seal. After peeling off the film, the contents of the wells could be dehydrated by heating to 95 °C for 5 min.

The reagents for the next procedure were added on top of the previously dehydrated well components. To ensure that the reagents did not exceed the volume of the wells, the water in the reagents was allowed to evaporate at standard room conditions for approximately 10 min until the meniscus of the fluid inside the wells became concave by visual inspection. The wells were then sealed with a fresh Kapton film.

We estimate that resulting variations in dilution are at most 5%, the same as that for commercial 96-well PCR plates. It should also be noted that the evaporation of fluid would not affect the yes/no detection of DNA at the levels reported here.

DNA amplification protocol

To perform PCR amplification, PCR reagents were added to wells that had previously been dehydrated. Standard PCR mastermix (Invitrogen 10572-014) was adjusted to 0.1% wt/v BSA

(Fisher BP675-1), 60 U mL^{-1} Taq DNA polymerase (ABgene AB-0301a), and 2.75 mM $MgCl_2$ (ABgene AB-0301a). Primers for a GAPDH 167 bp genomic DNA target (5′catcatctctgccccctct and 5′tgagtccttccacgatacca, unless otherwise specified) were added to a final concentration of 200 nM in the PCR supermix. The device was sealed with Kapton film, compressed, and then thermocycled. PCR thermocycling was performed at 95 °C for 2 min followed by 35 cycles of 95, 56, and 72 °C for 10, 10, and 15 s, respectively, followed by a final 72 °C step for 2 min and a 0 °C step for 10 min.

Visualization

DNA-intercalating dye was used to visualize the PCR amplification products. The top sealing film was removed and 300 μL of a 10× dilution of SYBR Green I dye (Fisher Scientific BMA50513) were added to the top surface of the device and allowed to diffuse into the agarose for 5 min. SYBR Green I is an intercalating dye that stains double-stranded DNA. The dye solution was rinsed off to reduce background noise. The device was placed on a blue light transilluminator (Clare Chemical DR45M) and imaged with a CCD camera (Nikon D50) with a shutter speed of 1/15 of a second. An amber screen included with the transilluminator was placed between the CCD and the device.

Results

Direct DNA extraction and amplification: protocols and validation

New protocols were developed to isolate DNA from tissue and then to proceed directly to PCR amplification in the wells of the device. This "single-tube" approach simplifies the currently used process of detecting genes from tissue, which requires centrifugation and sample pipetting between multiple tubes. These techniques were initially validated because the device, protocols, and reagents were new. Also, the inclusion of 2% agarose and the small aluminium wells created a novel environment for PCR that necessitated a comprehensive evaluation of system performance.

DNA extraction protocol. DNA was extracted directly using water and proteinase K, since the carry over of additional buffer salts is known to inhibit PCR reactions. To prepare the wells for the addition of extraction solution, the agarose and tissue were dehydrated within the device using the heating rig at 95 °C for 5 min. To isolate the DNA, the cells were lysed in water mixed with proteinase K (Invitrogen 25530-049) at 2 mg mL^{-1} (extraction solution); 2.4 μL of this solution were manually pipetted into each well. To prepare for device sealing, the reagents were dehydrated at room temperature for 10 min and then frozen at −20 °C for 10 min. The device was sealed and heated to 65 °C for 30 min to digest the tissue and at 95 °C for 5 min to inactivate the enzyme. The well contents were then solidified and dehydrated.

Validation of DNA extraction protocol. The direct DNA extraction protocol was tested using frozen normal prostate tissue that had been snap frozen to a glass slide. Tissue scrapes were placed into 12 randomly chosen wells. The tissue was diluted to 6.7 ng μL^{-1} in the extraction solution. The tissue–proteinase K mixture was subjected to the DNA extraction protocol, except without the addition of agarose.

Controls were then added to other wells to semi-quantitatively gauge the efficacy of the direct DNA extraction procedure. First, genomic DNA (BioChain Institute D1234106) was added as a dilution series of 1.06 ng μL^{-1}, 212 pg μL^{-1}, and 42 pg μL^{-1}, which represents a range of easily detectable to non-detectable levels of starting material. Water was also added to provide a negative control. The contents of the wells were dehydrated as described previously and subjected to PCR. Validation was performed by gel electrophoresis at 100 V for 35 min in a 2% gel (NuSieve 3 : 1) containing 1× TBE and 1× SYBR-Gold dye to see if the 167 bp target DNA was amplified.

An electrophoresis gel (see ESI† for a photo of the gel) showed a 167 bp DNA fragment from both purified genomic DNA controls above 212 pg μL^{-1} and from prostate tissue, but not from the negative control. Furthermore, the observed amplified bands from prostate tissue were of identical size to those from genomic DNA owing to the specificity of PCR. These results demonstrate that the frozen prostate tissue was lysed and the DNA was amplified in the multi-well device without additional purification steps.

Validation of DNA amplification protocol. To test that PCR was reliable and robust in the agarose-filled aluminium miniature well environment, a variety of amplifications were carried out simultaneously in 12 wells of the device. Two templates (sources of DNA) were used.

1. Human OsteoSarcoma cDNA at 4.9 ng μL^{-1} made from mRNA (Ambion Inc. AM7868) by a standard reverse transcription protocol.

2. A 582 bp PCR target amplified in a thin-walled PCR tube to 5×10^9 molecules μL^{-1}.

Four sequences on the first template were targeted, and the second template was diluted to four different concentrations. The PCR products were run on an electrophoresis gel (see ESI†). All the observed gel bands corresponded to primers added to the wells.

In these results a well filled only with PCR supermix was immediately adjacent to a well that contained previously amplified PCR targets at a high concentration. The fact that no bands were observed from this negative control well demonstrates that there was no cross-contamination between wells. The PCR amplification was effective for both purified targets and mixtures containing many non-specific targets. Taken together, these results demonstrate that PCR within the wells robustly amplifies a variety of targets from a variety of templates including crude genomic DNA, crude cDNA, and PCR-amplified products.

2D-PCR mapping

To validate the concept of 2D-PCR, the presence or absence of specific DNA sequences in tissue sections was mapped as a first step towards the more desirable quantification of DNA by real-time PCR methods. To this end, a genomic DNA target sequence (GAPDH) in a breast tissue section was mapped and correlated with the known geometry of the histology. The GAPDH gene is present in the nuclear DNA sequence of every cell, and is thus

found throughout the tissue. The map would therefore be expected to show where the tissue was present above the device. Using the 2D-PCR approach, a GAPDH genomic DNA target from breast tissue was extracted, PCR-amplified, and visualized by SYBR Green I with 1.6 mm resolution.

2D-PCR mapping protocol. To identify the tissue area visually, the sample was stained pink with Eosin Y. The tissue was transferred onto the device surface and imaged (Fig. 3). It was then pressed into the wells and dehydrated. The tissue was then lysed to release the DNA as described in the DNA extraction protocol, with the proteinase K concentration reduced to 1 mg mL^{-1}. To indicate the orientation of tissue on the device, a diagonal and square notch were cut out of the sealing film covering the tissue. After dehydration, the GAPDH target region of genomic DNA was amplified. After PCR, the sealing film was removed.

SYBR Green I dye was added. Solidifying the DNA in agarose enabled the DNA to be stained with SYBR Green I dye without losing the DNA from the wells. The fluorescent image of the device is shown in Fig. 3. Those wells that were under the tissue fluoresced, demonstrating that the DNA had been amplified to sufficiently high levels. The wells that were completely under the tissue fluoresced the most. The negative control area, which was not covered by tissue, had only a low fluorescent background signal. The detection signal correlated with the known area of the tissue, demonstrating that the 2D-PCR approach was successful.

Validation of 2D-PCR. To validate that the fluorescent signals correlated with the desired target 167 bp GAPDH PCR product, the well contents were run on an electrophoresis gel. To do this, the device was cooled to 0 °C for 10 min to solidify the agarose, and the top sealing film was removed. A pipette (Fisher 02-707-439) was used to obtain gel plugs from six fluorescent and six non-fluorescent wells. Each plug was diluted 100 times with water and melted at 95 °C for 10 min to dilute the Eosin dye. Then, to offset the dilution, each sample was amplified by 8 cycles of PCR (110-fold amplification at 90% efficiency) using the same PCR conditions as previously described. Samples were subjected to electrophoresis at 100 V for 35 min in a 2% gel containing 1× TBE and 1× SYBR-Gold dye.

A signal corresponding to the 167 bp GAPDH genomic DNA target was observed in lanes 1–6, which correlates with the presence of tissue (Fig. 4). There were no observed false positives from wells without tissue (lanes 7–12). Note that the negative well 9 was directly adjacent to wells covered by tissue, yet there was no observed cross-contamination. These results confirm that the 2D-PCR method can be used to isolate tissues, extract DNA, amplify DNA, and visualize DNA in a spatially resolved manner for tissue sections.

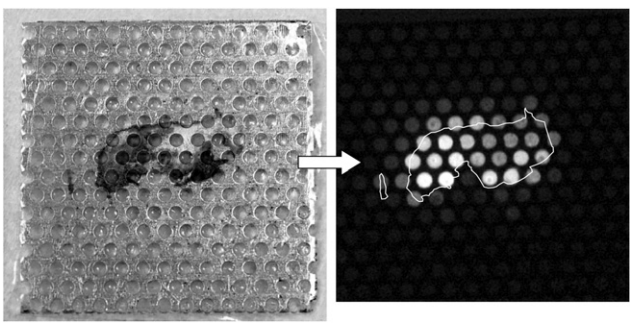

Fig. 3 Image of a breast tissue section (stained for visualization with Eosin Y) after transfer onto the multi-well device, and the visualization map created by fluorescent DNA detection with SYBR Green I after amplification by 2D-PCR. The tissue position is indicated by the outline.

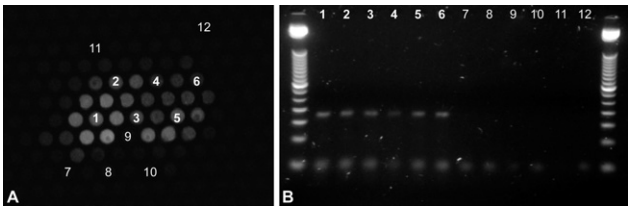

Fig. 4 (A) The wells chosen for electrophoretic validation of the fluorescent signal. (B) In wells containing tissue (lanes 1–6) there was a bright fluorescent signal corresponding to detection of a 167 bp GAPDH genomic product, while negative wells (lanes 7–12) had no amplification of the 167 bp target and only weak bands of ~50 bp primer dimers, an artifact from PCR.

Preliminary work towards miniaturization

A longer term goal of this research is to create a tool with well sizes of 50–100 μm, which would produce 2D maps with a resolution of ~100 cells. Such miniaturization has challenges associated with microfabrication, sample loading, rapid fluid evaporation, lower amplification efficiency, and smaller volumes for fluorescence visualization. We have performed some preliminary studies to identify a path to miniaturization. We describe these steps briefly here and give further details in ESI†.

Standard microfabrication with deep reactive ion etching (DRIE) in silicon allows the realization of through-hole wells that are tens of μm in diameter. Multi-well arrays were fabricated in Si using DRIE. They had wells that were 100 μm in diameter and 400 μm deep (the thickness of the wafer); they were spaced 100 μm apart, yielding 2500 wells cm^{-2} (Fig. 5).

It is not possible to pipette reagents into such small wells, but fluid can be pulled into the wells by capillary forces if a wetting agent is added to make the etched walls more hydrophilic. Placing a droplet of water containing BSA, Triton X-100, or Tween 20 of an appropriate concentration (see ESI†) onto one side of the micro-well plate and allowing it to dry resulted in subsequent complete filling of the wells by both aqueous solutions (Fig. 5) and agarose solutions up to concentrations of 1.5%. None of these surface treatments inhibited PCR, consistent with prior work.[29,30]

The micro-wells must be sealed to prevent spreading and/or evaporation of reagents. This requires a material that conforms to surface variations of the plate to create a good seal, yet that at the same time is not so viscoelastic and adhesive that the sealing material embeds itself permanently into the holes, preventing reversible sealing. Microseal A (MJ Research) was found to prevent evaporation of reagents from most of the micro-well plate, and it also prevented 99.8% of cross-talk (ESI†).

To demonstrate PCR in the micro-well plate, the surfaces were pretreated with BSA and loaded by capillary action with a PCR

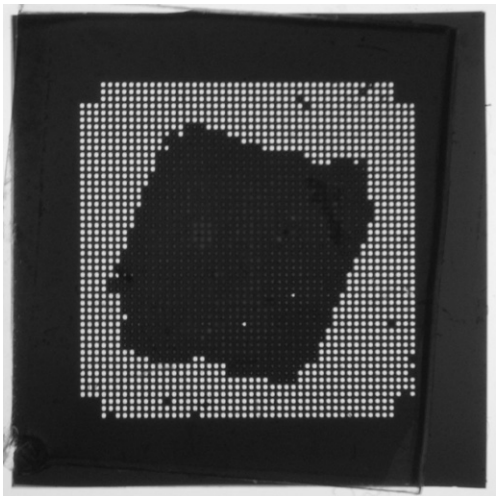

Fig. 5 Silicon substrate having a 1 cm^2 area with 100 μm diameter holes spaced 100 μm apart. The surface was pretreated with BSA and spotted with water containing food dye for visualization (dark area in center). The wells filled completely by capillary action.

mastermix containing a primer set and cDNA that had been used previously in the mini-vials, as well as additional native Taq DNA polymerase and additional BSA. The additional BSA is needed with large surface to volume ratios to prevent non-specific adsorption on the walls of the wells. For the negative control, water was substituted in place of cDNA. No gel was used in the wells here, so preventing evaporation was particularly important; the micro-well plate was sealed using Microseal A. The micro-well plate was placed on the flat alpha unit block of the thermocycler, covered with mineral oil to maximize thermal contact, and thermally cycled. For validation, the fluid within the vials was recovered and combined, and loaded onto a 1.5% agarose electrophoresis gel. The gel results (ESI†) verified successful PCR.

Discussion

Several challenges in using the multi-well array approach were overcome to obtain the DNA mapping results presented here. The first was transferring tissue into the device while segregating tissue subregions (or "pixels") to preserve overall spatial information. The second was creating a new single-well protocol for extracting the DNA, amplifying the DNA, and detecting the amplified DNA. The third was creating a new technique for sample containment to prevent cross-contamination and evaporation between wells. The current 2D-PCR technology is now ready for optimization to study specific alterations in DNA, such as LOH, SNPs, and methylation using methylation-specific PCR.[31]

Lab-on-a-chip devices typically use microfluidics on a planar surface to interconnect processing chambers for isolating and amplifying DNA from cells,[32–34] but this consumes too much space on the chip surface to map DNA from tissues with practical density. The single-well procedure for processing samples eliminates the need for fluid movement while providing direct surface access to capture as much tissue as possible.

The first challenge was addressed by loading the tissue vertically, pressing it directly into the array of wells to isolate tissue subregions while preserving the overall 2D spatial relationship. Even though tissue remained on the top of the device between the wells, the 2D-PCR results demonstrated that this tissue does not contribute a signal to the adjacent wells. This tissue loading technique also has the advantage of beginning with traditional histological sectioning and transfer steps, which are familiar to investigators and clinicians who may wish to adopt this technique for research or diagnosis.

A new approach was used to extract DNA from tissue, amplify it, and detect the amplification products with fluorescence visualization. We used a direct DNA extraction protocol so that the reagents could be inactivated and dried without inhibiting subsequent PCR. Afterward, the PCR products were stained directly with a fluorescent DNA-intercalating dye. This approach streamlines the protocols used in commercially available kits, which require centrifugation and re-pipetting of each sample between three or four tubes.[35–39] Performing the processes sequentially in a single well not only enables a simple device geometry but is also amenable to further miniaturization. Others have also realized the importance of simplifying and/or miniaturizing commercially available nucleic acid processing protocols by using fewer steps, tubes, or reagents,[32–34,40–48] but those techniques do not make it possible to map DNA from tissue.

Directly visualizing the PCR products by staining them with fluorescent dye allows them to be detected across the entire array, creating 2D maps both efficiently and directly *in situ*. There is sufficient DNA in an 8 μm thick tissue sample for detection in a single 1.6 mm diameter well. Given the facts that there were at most 5000 cells captured per well, that the detection signal was strong, and that detection occurred at 35 cycles, it is anticipated that 2D-PCR can be extended to the level of about 100 cells per well—a limit we expect due to the efficiency of DNA extraction with this method rather than PCR sensitivity (which has been shown to amplify DNA even from single cells[49]). In the future, it would also be desirable to use real-time PCR to detect the PCR products, eliminating the staining step, as others have demonstrated.[50] It is also possible to detect the products by fluorescent hybridization after blotting onto detection membranes.[27]

The single-well protocol depended on a new reversible sealing procedure to contain the samples across an array format and to enable the addition of successive reagents. Without sealing, reagents can evaporate or spread during the heating steps. The vapor was contained within the wells by applying compression to a sealing film placed on top of the entire multi-well array. Suppliers of 96-well or denser multi-well plates also employ reversible seals to prevent evaporation of the water, but this is the first example of reversible sealing across an array of holes in a flat surface. PCR lab-on-a-chip reactors typically employ irreversible seals, such as nail polish or epoxies, to attach glass cover slips onto silicon PCR wells.[11,29,50] Reversible sealing was used for 2D-PCR because different reagents needed to be added and dehydrated at different times. This method of sealing was successful with both the aluminium and the silicon substrates.

Sample containment also relied on the addition of 2% agarose gel to the wells before the tissue transfer step to prevent the movement of fluid and to immobilize the DNA. PCR has not previously been reported in the presence of greater than 0.5%

agarose, although there have been reports of up to 3% agarose used with other biological enzymes.[28] We note that it is also possible to prevent mixing by freezing the fluids prior to seal removal, but the resulting condensation can prevent future sealing.

It is also of interest to miniaturize the device presented here to provide biomolecular information at the level of a few dozen cells, which would allow pathologists to overlay 2D genetic maps over a tissue histology and see molecular variations as a function of cell type. We have solved some of the challenges of such miniaturization, but significant challenges still need to be overcome. The greatest challenge will be transferring tissue into the micro-wells in such a way that the tissue does not block the openings, preventing future transfer of fluid. Solutions to this issue may include electro-transfer of DNA from tissue lysates or using a more disruptive tissue lysing process. Another major challenge is designing a batch fluid-loading system that is consistent, prevents cross-contamination between wells, and avoids uneven evaporation.

We are currently working on mapping RNA from tissue, since current visualization techniques for RNA cannot reliably detect below a few dozen copies per cell *in situ*.[24,51] There are many techniques that can currently detect high-abundance targets directly in a tissue section, but based on recent publications and personal accounts of researchers in the field, these techniques have failed for lower-abundance targets because they are either unreliable, difficult to reproduce, or too time-consuming. On the other hand, the robustness of LCM has led to widespread acceptance because it can be used to study small cell populations with reliable down-stream techniques. Unfortunately, LCM is only employed to study a limited number of cell populations because of time and cost constraints. Thus, a promising new way to address the need for mapping low-abundance RNA targets is *via* the 2D-PCR method presented here.

2D-PCR opens several other promising avenues for technical research. One ideal 2D-PCR development would be the ability to study formalin-fixed paraffin-embedded (FFPE) tissue samples in addition to frozen samples. FFPE tissue represents the majority of clinical tissue samples available for study, but these samples contain fragmented and immobilized RNA due to the formalin cross-linking. The technique could also be extended to other 2D samples, such as Southern blot gels. Finally, in the future, it would also be desirable to use quantitative real-time PCR (qPCR) to detect the PCR products, eliminating the staining step as others have demonstrated.[50] To account for variations in the number of cells loaded, the PCR would need to be multiplexed to include housekeeping genes to which other signals could be normalized. However, qPCR machines that would be compatible with the setup presented in this work are not commercially available and would require a significant effort to customize.

Currently, biological research is growing with respect to the number of genes and number of patients studied, but it suffers from slow growth with respect to comprehensive tissue analysis. Therefore, a potentially promising avenue for future work is facilitating human genetic anatomy research that would create gene expression maps of human tissue. This can be applied to study tissue heterogeneity and tumor microenvironments, to map the extension of the molecular field effect from cancer into normal regions (the "histological margins of tumors" *vs.* the "molecular margins", which can have potential clinical use in the surgical resection of neoplasias), and to the study of the molecular anatomy of normal and pathological tissues.

It would be possible to use the 2D-PCR method to create 3D maps of tissue. By combining information from a number of serial cut tissue sections taken from a single tissue block, one can create 3D maps, as shown in ref. 16. To facilitate this process, the sample handling should be automated. The practicality of 3D mapping will depend on how quickly, easily, and cheaply the 2D-PCR can be performed, a goal for future work.

The spatial genetic data may also elucidate the normal function of human genes, which is known for only 30% of them.[52] To highlight the interest in mapping gene function in tissues, there have been several initiatives for procuring and archiving gene expression images on the mouse, including BGEM,[53] Brainatlas,[54] Genepaint,[55] GENSAT,[56] Mamep,[57] and EMAGE.[58]

Conclusions

We have developed a novel approach for creating maps of DNA preserving the 2D architecture of the tissue based on extracting the nucleic acids from a tissue section and then amplifying them within a controlled environment in such a way that the positional information is preserved. To validate this method, we demonstrated that DNA could be mapped from a frozen human tissue section, a result that represents over a hundred successful DNA isolations and subsequent PCR reactions performed in parallel on a single device, in less than a few hours total time.

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