Temperature-controlled microintaglio printing for high-resolution micropatterning of RNA molecules

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Abstract

We have developed an advanced microintaglio printing method for fabricating fine and high-density micropatterns and applied it to the microarraying of RNA molecules. The microintaglio printing of RNA reported here is based on the hybridization of RNA with immobilized complementary DNA probes. The hybridization was controlled by switching the RNA conformation via the temperature, and an RNA microarray with a diameter of 1.5 μm and a density of 40,000 spots/ mm^2 with high contrast was successfully fabricated. Specifically, no size effects were observed in the uniformity of patterned signals over a range of microarray feature sizes spanning one order of magnitude. Additionally, we have developed a microintaglio printing method for transcribed RNA microarrays on demand using DNA-immobilized magnetic beads. The beads were arrayed on wells fabricated on a printing mold and the wells were filled with *in vitro* transcription reagent and sealed with a DNA-immobilized glass substrate. Subsequently, RNA was *in situ* synthesized using the bead-immobilized DNA as a template and printed onto the substrate via hybridization. Since the microintaglio printing of RNA using DNA-immobilized beads enables the fabrication of a microarray of spots composed of multiple RNA sequences, it will be possible to screen or analyze RNA functions using an RNA microarray fabricated by temperature-controlled microintaglio printing (TC- μIP).

Keywords

Microarray, Intaglio printing, RNA patterning, *In situ* synthesis, Parallel spotting, High-throughput screening

Main Text

1. Introduction

Recently, both the understanding and the use of the functions of RNA have become increasingly important in biology and related fields. In the last two decades, it has been revealed that noncoding RNAs such as micro-RNA, small interfering RNA, and riboswitches play important roles in various biological phenomena including development, differentiation, and metabolism by regulating gene expression (Aalto and Pasquinelli, 2012; Serganov and Nudler, 2013). Furthermore, artificial RNAs such as aptamers and ribozymes have been developed and used in various fields including therapy and diagnostics (Radom et al., 2013; Mulhbachter et al., 2010), synthetic biology (Khalil and Collins, 2010), and biosensors (Tombelli et al., 2005; Sassolas et al., 2011). To comprehend the functions of these RNAs and promote their use, it is reasonable to employ high-throughput analysis technology such as microarrays for measuring the functions of highly diverse natural and artificial functional RNAs (Collett et al., 2005).

Microarrays such as DNA and protein/peptide microarrays are commonly used for the analysis of expression profiles, DNA-protein interactions, epitopes, and so forth. In addition to these microarrays, RNA microarrays (i.e., RNA-immobilized microarrays) are a powerful tool for analyzing RNA-related phenomena, even though they have not been conventionally used. RNA aptamer microarrays for detecting DNA molecules (Goodrich et al., 2004a, 2004b; Lee et al., 2005) and proteins (Chen et al., 2012; Cho et al., 2006; Collett et al., 2005; Li et al., 2006, 2007) and ribozyme microarrays for detecting various target molecules (Seetharaman et al., 2001) have been reported. The RNA microarray fabrication methods used in previous studies can be divided into three types. The first involves the direct attachment of chemically modified RNA onto a substrate such as the immobilization of biotinylated RNA onto a streptavidin-coated substrate (Cho et al., 2006; Collett et al., 2005) and thiol-modified RNA onto a gold-coated or maleimide-functionalized substrate (Goodrich et al., 2004a, 2004b; Li et al., 2007; Seetharaman et al., 2001). The second is a method in which RNA is arrayed via the assistance of a previously fabricated DNA

microarray. Corn and co-workers fabricated an RNA microarray by ligating RNA with prearrayed DNA (Lee et al., 2005; Li et al., 2006). Kim and Crooks reported a method in which RNA and a biotinylated DNA oligomer were hybridized on prearrayed DNA, and the RNA was biotinylated by ligation of the biotinylated DNA oligomer on the prearrayed DNA, which was followed by the immobilization of the biotinylated RNA onto a streptavidin-modified substrate to fabricate an RNA microarray (Kim and Crooks, 2007). The third is a method in which an RNA microarray is fabricated using on-chip-synthesized RNA as reported by Chen et al. (Chen et al., 2012). In this method, DNA is arrayed on a microfluidic channel, and RNA is transcribed on demand from the DNA and hybridized with another DNA prearrayed downstream of the channel. There are specific advantages of the latter two methods, they avoid the tedious chemical modification of RNA and enable the parallel arraying of RNA, and the third method can use fresh intact RNA molecules synthesized on demand.

We have previously reported the use of microintaglio printing (μ IP) (Biyani et al., 2011; Biyani et al., 2013) to fabricate a high-density microarray pattern of biological molecules. In μ IP, biological molecules such as proteins are intaglio-printed on a substrate using a microfabricated printing mold. Since μ IP enables parallel *in situ* synthesis and the immobilization of biological molecules, the need for chemical modification and the risk of degradation of labile molecules, such as RNA, can be reduced. However, the high background noise (i.e., the low contrast between the foreground (spots) and background) remains the most challenging issue in μ IP, particularly for printing a ready-to-print ink molecule. Owing to the nature of μ IP processing, there is a risk of an ink molecule (i.e., an RNA molecule in this report) remaining in the nonarray region (outside the grooves on the printing mold) while achieving a tight seal between the micromold and the substrate. Thus, part of the ink molecule could also be undesirably printed on the substrate, leading to low-contrast printing. In principle, there are three approaches to accomplishing high-contrast μ IP while avoiding the undesired adsorption and/or printing of an ink molecule on a substrate in the nonarray region. (1) restricting the type of ink molecule to one with a low adsorption rate on the substrate, (2) allowing the *in*

situ synthesis of an ink molecule only inside the mold region (Biyani et al., 2013), and (3) controlling the adsorption and/or printing reaction of the pre-synthesized ink molecule via the temperature or a light trigger. Acceptable printing contrast has been accomplished in our previous studies by approach (1) using relatively long chain RNA molecules (Biyani et al., 2011; Ueno et al., 2012) and approach (2) using an *in situ* synthesized ink molecule (Biyani et al., 2013). Here, we studied the use of temperature-controlled μ IP (TC- μ IP), which is an advanced form of μ IP based on approach (3), to fabricate a fine micropattern of RNA molecules with high density and high contrast. TC- μ IP is based on a controllable hybridization reaction between presynthesized RNA and a substrate-immobilized complementary DNA probe by switching the ability of the RNA to fold into a secondary structure via the temperature during μ IP processing. We also present an RNA microarray fabrication method for array-on-demand purposes by TC- μ IP based on the *in situ* synthesis of RNA molecules using bead-immobilized DNA on a printing mold.

2. Experimental section

2.1. Overview of TC- μ IP procedure

Figure 1 shows a schematic of the TC- μ IP method used for RNA patterning in this study. Presynthesized RNA solution was applied on a microfabricated printing mold made of polydimethylsiloxane (PDMS) and pressed with a gold-coated glass substrate modified with a thiolated single-strand DNA (ssDNA) oligomer (capture DNA), whose sequence was complementary to the terminus sequence of the RNA. The RNA was denatured and hybridized with the capture DNA during the press step, and an RNA micropattern was fabricated on the substrate as a result. The hybridization can be controlled via the temperatures T_1 and T_2 during the ink application and press steps, respectively, and the incubation time at T_2 (defined as t). The hybridization is controlled by thermal denaturation of the RNA. First, an interstrand structure of the RNA was formed at a low temperature T_1 to prevent premature hybridization with the capture DNA then thermal denaturation was conducted by a relatively long incubation at a high temperature T_2 to promote hybridization after complete press-sealing. After the denaturation step, the denatured RNA was hybridized with the capture DNA by gradual cooling to room temperature.

2.2. Preparation of DNA and RNA

Thiol-modified capture DNA, 5'-HS-(CH₂)₆-TTTTTTTTTTTTTTTTTTTTAGATGTCTCGTAGTAGTCGC-3', and thiol- and Cy5-modified DNA, 5'-HS-(CH₂)₆-TTTTTTTTTTTTTTTTTTTTTTTTTTTTTTT-AGATGTCTCGTAGTAGTCGC-[Cy5]-3', were custom-synthesized by Nihon Bio-Service (Japan) using general phosphoramidite chemistry. A double-strand

DNA (dsDNA) construct (851 bp) encoding T7 promoter, green fluorescent protein (GFP), and the hybridization sequence of the capture DNA was constructed by overlap extension polymerase chain reaction (PCR). Cy3- or Cy5-modified RNA (815 mer) encoding GFP and the hybridization sequence of the capture DNA was prepared by *in vitro* transcription using the T7 RiboMAX Express Large Scale RNA Production System (Promega, USA) supplemented with Cy3-UTP and Cy5-UTP (GE Healthcare, USA) using the DNA construct as a template in accordance with the manufacturer's instructions.

2.3. Preparation of printing mold

A printing mold made of PDMS was prepared by a general soft-lithography process (Xia and Whitesides, 1998; Gates et al., 2005). An SU-8 convex master mold was fabricated as follows: a silicon wafer was coated with hexamethyldisilazane (OAP, Tokyo Ohka Kogyo, Japan) using a spin coater and baked at 65°C. Subsequently, the wafer was coated with a negative photoresist (SU-8, Nippon Kayaku, Japan) using a spin coater and baked at 65°C. The wafer was exposed to UV light through a patterned mask using a mask aligner (PEM-800; Union Optics, Japan) and baked at 65°C and 95°C, which was followed by development using 1-methoxy-2-propyl acetate (SU-8 developer, Nippon Kayaku, Japan). A mixture of PDMS prepolymer and catalyst (Silpot 184; Dow Corning Toray Silicone, Japan) was cast onto the master mold and cured by heating at 100°C. The polymerized PDMS was peeled off from the master mold.

2.4. Preparation of DNA-modified gold substrate

The gold substrate was prepared as follows: a cover glass (20 mm × 20 mm × 0.5 mm; Matsunami Glass, Japan), which was preliminarily cleaned with sulfuric acid and hydrogen peroxide mixture (SPM), was successively coated with a 10-nm-thick chrome layer and 200-nm-thick gold layer by sputtering. The gold

substrate was cleaned with SPM and the surface of the substrate was covered with 1 μ M thiol-modified capture DNA in 1 $\times$ phosphate buffered saline (PBS) and incubated for 24 h in a sealed chamber. The substrate was dipped in 1 mM 6-mercapto-1-hexanol (MCH) in 1 $\times$ PBS and incubated for 1 h. The DNA-modified gold substrate was washed successively with 1 $\times$ PBS with 0.2% Tween 20, 1 $\times$ PBS, and water, and finally dried.

2.5. Microintaglio printing of pre-synthesized RNA

The printing mold was immersed in 0.1% sodium dodecyl sulfate (SDS) for 30 min to hydrophilize its surface. The hydrophilized PDMS mold was attached to a glass slide and placed on the heat-controllable flat metal plate of a thermal cycler preset to 4°C, 25°C, or 70°C. 10 μ l of 1 μ M Cy3- or Cy5-labeled RNA in 3 $\times$ SSC buffer (1 $\times$ SSC buffer is 0.15 M sodium chloride, and 0.015 M sodium citrate) with 0.02% SDS was applied onto the surface of the printing mold and pressed with the DNA-modified gold substrate using a mass of 2 kg. After complete press-sealing, the metal plate was kept at 70°C for 1 to 120 min then cooled to 25°C with a cooling rate of 0.1°C/s to denature the RNA conformation and hybridize the RNA with the capture DNA on the gold substrate. The substrate was released from the printing mold and washed successively with 3 $\times$ SSC, 0.1 $\times$ SSC with 0.1% SDS, and 0.1 $\times$ SSC. The fluorescence of the Cy3-labeled RNA hybridized with the capture DNA on the gold substrate was visualized by confocal laser scanning microscopy (Digital Eclipse C1; Nikon, Japan) in combination with a 561 nm laser and a 570LP dichroic mirror. Cy5-labeled RNA hybridized with the capture DNA on the gold substrate was visualized by confocal laser scanning microscopy (LSM510; Zeiss, Germany) in combination with a 633 nm laser and a 655-710 nm band-pass filter.

2.6. Microintaglio printing of in situ synthesized RNA

1
2 A 851 bp biotinylated dsDNA was prepared by PCR using a biotinylated primer for the 5'-terminus of a
3 sense sequence. The biotinylated dsDNA was immobilized with streptavidin-coated magnetic beads with a
4 diameter of 2.8 μm (Dynabeads M-280 Streptavidin; Life Technologies) in accordance with the supplier's
5 instructions. The DNA-immobilized magnetic beads in a buffer (10 mM Tris-HCl, 1 mM EDTA, 2 M NaCl,
6 0.2% Triton X-100, pH 7.5) were applied on the printing mold, which had wells with a diameter and depth of
7 4 μm , and the beads were arrayed on the wells with a single bead in each well using magnetic force scanning.
8 After arraying the beads, the printing mold was washed with water, dried, and placed on the heat-controllable
9 flat metal plate of a thermal cycler precooled to 4°C. Ten microliters of an *in vitro* transcription reagent
10 supplemented with Cy5-UTP was applied onto the surface of the printing mold and pressed with the capture
11 DNA-modified gold substrate. After complete press-sealing, the metal plate was kept at 37°C for 120 min to
12 induce the *in vitro* transcription reaction. After the reaction, the metal plate was kept at 70°C for 10 min then
13 gradually cooled to 25°C to denature the *in situ* synthesized RNA and hybridize the RNA with the capture
14 DNA on the substrate. Finally, the substrate was released from the printing mold and washed successively
15 with 3 $\times$ SSC, 0.1 $\times$ SSC with 0.1% SDS, and 0.1 $\times$ SSC. The fluorescence of the Cy5-labeled RNA printed on
16 the gold substrate was visualized by confocal laser scanning microscopy in combination with a 633 nm laser
17 and a 655-710 nm band-pass filter.
18

3. Results and discussion

3.1. Temperature-controlled press-sealing

First, the effect of the press-sealing temperature (T_1) on the quality of the result of μ IP was examined. The purpose of introducing press-sealing temperature is to evaluate the effect of ‘switching-on/off’ the ink (i.e., RNA molecules) onto the signal-to-background ratios. This can be done by controlling the structure of large RNA molecules, i.e., ‘folded-state’ which unable the ink to hybridize (switching off) or ‘unfolded-state’ which able the ink to hybridize (switching on). For this purposes, we need at least two temperatures: low temperature to remain RNA molecules in folded-state and high temperature to turn the folded-state into unfolded-state which is required for the denaturation of secondary structures of RNA molecules and so thus to induce the hybridization reactions. The press step were performed at 4°C, 25°C, or 70°C (press-sealing temperature, T_1), and was followed by incubation at 70°C (ink-activating temperature, T_2) to denature the RNA. The temperature was then linearly decreased to 25°C (ink-printing temperature, T_3) at a cooling rate of 0.1°C/s to induce hybridization between the RNA and the capture DNA. The signal-to-background ratios of the printed patterns were approximately 2.0, 9.5, and 12.0 for $T_1 = 70^\circ\text{C}$, 25°C , and 4°C , respectively (Fig. 2). This result indicates that premature hybridization during the press-sealing step occurred at $T_1 = 70^\circ\text{C}$ because the RNA strand was already denatured and ready for hybridization, and this led to the low contrast. The contrast was improved at $T_1 = 25^\circ\text{C}$ and below by folding the RNA into secondary structures and thus preventing the hybridization reaction until all the ink molecules (RNAs) had been removed from the nonarray region during the press-sealing step.

Next, the effect of the incubation time t at $T_2 = 70^\circ\text{C}$ was examined. After the press step at $T_1 = 4^\circ\text{C}$, the printing mold pressed with the substrate was incubated at $T_2 = 70^\circ\text{C}$ for $t = 1$ -120 min, which was followed by gradual cooling to 25°C with a cooling rate of -0.1°C/s . As shown in Fig. 3, an incubation time of 10 min or longer was necessary for sufficient RNA denaturation and efficient hybridization. On the basis of these

results, the conditions of $T_1 = 4^\circ\text{C}$, $T_2 = 70^\circ\text{C}$, and t over 10 min were used in subsequent experiments.

3.2. Miniaturization of RNA spots

The miniaturization of printed patterns is necessary to fabricate high-density microarrays or fine micropatterns. Therefore, we investigated the effect of the size of the printed pattern in TC- μIP . A printing mold having wells with various diameters of 4-50 μm was fabricated and used for the investigation. As shown in Fig. 4a, RNA was printed with comparable efficiency for a range of well diameters spanning one order of magnitude. Then, we successfully fabricated an RNA microarray with a diameter of 1.5 μm and a density of 40,000 spots/ mm^2 by high-contrast printing with temperature control (Fig. 4b). These results clearly indicate that TC- μIP can be used for the miniaturization of arrays to obtain high-density nanoarrays while maintaining the high resolution of printing spots in the designated array region by minimizing the adsorption and/or printing of ink molecules in nondesignated region.

3.3. Printing of arbitrary pattern

To evaluate the robustness and utility of μIP for printing fine patterns other than array patterns, we fabricated a printing mold with a pattern of the letters “RNA” with a line width of 7.5 μm and successfully printed the pattern by TC- μIP using RNA molecules as shown in Fig. 5. This technique should be applicable to the fabrication of miniaturized patterns using RNA aptamers (Strackharn et al., 2012). The flexibility of TC- μIP allows the printing of any pattern of molecules with dimensions of μm order and thus TC- μIP can be applied for the solution-processed printing of biosensors and bioelectronics.

3.4. Printing of in situ synthesized RNA

1
2 In the above sections, TC- μ IP was performed using presynthesized RNA composed of a single type of
3 sequence.

4 In this section, we demonstrate an advanced form of TC- μ IP using DNA-immobilized magnetic beads and *in*
5 *situ* RNA synthesis (Fig. 6a). First, magnetic beads carrying a dsDNA encoding transcription promoter and
6 an RNA transcript sequence were arrayed onto a microwell array fabricated on a printing mold, as previously
7 reported for the production of a 60- μ m-scale array pattern of *in situ* synthesized protein biomolecules (Biyani
8 et al., 2013). In this work, a micromold plate with wells having a diameter and depth of 4 μ m was used.
9 Owing to the geometric constraint, superparamagnetic beads with a diameter of 2.8 μ m were used as carriers
10 to first capture the PCR-amplified dsDNA encoding the mRNA sequence by the single-emulsion PCR
11 technique (Diehl et al., 2006). Then the self-alignment of the beads on the micromold plate was induced
12 through an external dynamic magnetic force applied briefly by sliding a permanent magnet horizontally
13 along the bottom of the micromold plate. After arraying the beads, an *in vitro* transcription reagent was
14 applied on the wells and seal-pressed with the complementary DNA probe-bound substrate. The wells were
15 isolated from each other by press-sealing and the RNA was *in situ* transcribed in each isolated well from the
16 bead-immobilized dsDNA. Subsequently, the *in situ* synthesized RNA was hybridized with the capture DNA
17 and an RNA microarray pattern was fabricated on the substrate. The trigger of the *in situ* synthesis was also
18 controlled via the temperature, similarly to the hybridization. Using this advanced form of TC- μ IP, RNA
19 molecules composed of multiple sequences as well as those composed of a single type of sequence can be
20 arrayed on a single chip, making it possible to prepare an RNA microarray for the high-throughput screening
21 of RNA aptamers and ribozymes and the high-throuput analysis of RNA-protein interactions.

22 As a model experiment, a single type of biotinylated-dsDNA was immobilized on streptavidin-coated
23 magnetic beads. The dsDNA-immobilized beads were arrayed onto microfabricated wells at 4°C, and the *in*
24 *situ* synthesis of RNA in the isolated wells was conducted by incubation at 37°C for 120 min then, the *in situ*

1 synthesized RNA was printed on the substrate by denaturing at 70°C and gradual cooling to 25°C. As shown
2 in Fig. 6b, the RNA was successfully printed on the substrate only at the bead-containing wells without
3 crosstalk between the wells. In this procedure, the *in situ* synthesis of the RNA after the complete
4 press-sealing of the wells also contributes to the high-contrast printing. Additionally, this procedure enables
5 the immobilization of *in situ* synthesized “fresh” intact RNA and the sequence information of the RNA of
6 interest can be gained by sequencing of the bead-immobilized DNA in the corresponding well.

7 Conventional DNA microarrays fabricated by a robotic spotter (Hughes et al., 2001) or photolithographic
8 DNA synthesis (Pease et al., 1994) have a number of immobilized DNA spots on a single substrate limited to
9 10^5 order, which is too low to screen the huge diversity in aptamer libraries with up to 10^{15} variants generally
10 used in aptamer screening (Wilson and Szostak, 1999). However, the microarray format is advantageous to
11 the batch format for the screening of biomolecules (Emili and Cagney, 2000), and therefore the screening of
12 DNA aptamers using a DNA microarray has been attempted by several groups (Asai et al., 2005; Knight et al.,
13 2009). Since the massively parallel DNA arraying method employing the DNA-immobilized magnetic beads
14 used in this advanced TC-μIP enables the arraying of over 10^8 variants (Barbee and Huang, 2008), the
15 combination of this DNA arraying method, *in situ* RNA synthesis, and TC-μIP will enable the microarray
16 format screening of RNA aptamers and ribozymes in addition to DNA aptamers.

4. Conclusion

We have developed a method of fabricating a fine micropattern of RNA with high density and high contrast by controlling the timing of the initiation of RNA hybridization via a thermal trigger to switch the RNA conformation. Using this method, a fine pattern of microarray spots with a diameter of 1.5 μm and a density of 40,000 spots/ mm^2 and the printing of an arbitrary shape, applicable to the fine patterning of RNA, were demonstrated. Moreover, a microarray patterning method enabling the fabrication of multiple RNA sequence spots in parallel with high density was also developed by temperature-controlled *in situ* RNA synthesis. This method is expected to enable the measurement of the functions of various RNAs on a single chip with high throughput.

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References

- Aalto, A.P., Pasquinelli, A.E., 2012. *Curr. Opin. Cell Biol.* 24, 333-340.
- Asai, R., Nishimura, S.I., Aita, T., Takahashi, K., 2005. *Anal. Lett.* 37, 645-656.
- Barbee., K.D., Huang, X., 2008. *Anal. Chem.* 80, 21492154.
- Biyani, M., Osawa, T., Nemoto, N., Ichiki, T., 2011. *Appl. Phys. Express* 4, 047001.
- Biyani, M., Moriyasu, J., Tanaka, Y., Sato, S., Ueno, S., Ichiki, T., 2013. *Appl. Phys. Express* 6, 087001.
- Chen, Y., Nakamoto, K., Niwa, O., Corn, R.M., 2012. *Langmuir* 28, 8281-8285.
- Cho, E.J., Collett, J.R., Szafranska, A.E., Ellington, A.D., 2006. *Anal. Chim. Acta* 564, 82-90.
- Collett, J.R., Cho, E.J., Lee, J.F., Levy, M., Hood, A.J., Wan, C., Ellington A.D., 2005. *Anal. Biochem.* 338, 113-123.
- Diehl, F., Li, M., He, Y., Kinzler, K.W., Vogelstein, B., Dressman, D., 2006. *Nat. Methods.* 3, 551-559.
- Emili, A.Q., Cagney, G., 2000. *Nat. Biotechnol.* 18, 393-397.
- Gates, B.D., Xu, Q., Stewart, M., Ryan, D., Willson, C.G., Whitesides, G.M., 2005. *Chem. Rev.* 105, 1171-1196.
- Goodrich, T.T., Lee, H.J., Corn, R.M., 2004a. *J. Am. Chem. Soc.* 126, 4086-4087.
- Goodrich, T.T., Lee, H.J., Corn, R.M., 2004b. *Anal. Chem.* 76, 6173-6178.
- Hughes, T.R., Mao, M., Jones, A.R., Burchard, J., Marton, M.J., Shannon, K.W., Lefkowitz, S.M., Ziman, M., Schelter, J.M., Meyer, M.R., Kobayashi, S., Davis, C., Dai, H., He, Y.D., Stephanians, S.B., Cavet, G., Walker, W.L., West, A., Coffey, E., Shoemaker, D.D., Stoughton, R., Blanchard, A.P., Friend, S.H., Linsley, P.S., 2001. *Nat. Biotechnol.* 19, 342-347.
- Khalil, A.S., Collins, J.J., 2010. *Nat. Rev. Genet.* 11, 367-379.
- Kim, J., Crooks, R.M., 2007. *Anal. Chem.* 79, 8994-8999.
- Knight, C.G., Platt, M., Rowe, W., Wedge, D.C., Khan, F., Day, P.J., McShea, A., Knowles, J., Kell, D.B., 2009. *Nucleic Acids Res.* 37, e6.

- 1 Lee, H.J., Wark, A.W., Li, Y., Corn, R.M., 2005. *Anal. Chem.* 77, 7832-7837.
- 2 Li, Y., Lee, H.J., Corn, R.M., 2006. *Nucleic Acids Res.* 34, 6416-6424.
- 3 Li, Y., Lee, H.J., Corn, R.M., 2007. *Anal. Chem.* 79, 1082-1088.
- 4 Mulhbachter, J., St-Pierre, P., Lafontaine, D.A., 2010. *Curr. Opin. Pharmacol.* 10, 551–556.
- 5 Pease, A.C., Solas, D., Sullivan, E.J., Cronin, M.T., Holmes, C.P., Fodor, S.P., 1994. *Pros. Natl. Acad. Sci.*
6 USA. 91, 5022-5026.
- 7 Radom, F., Jurek, P.M., Mazurek, M.P., Otlewski, J., Jeleń, F., 2013. *Biotechnol. Adv.* 31, 1260–1274.
- 8 Sassolas, A., Blum, L.J., Leca-Bouvier, B.D., 2011, *Biosens. Bioelectron.* 26, 3725-3736.
- 9 Seetharaman, S., Zivarts, M., Sudarsan, N., Breaker, R.R., 2001. *Nat. Biotechnol.* 19, 336-341.
- 10 Serganov, A., Nudler, E., 2013. *Cell* 152, 17–24.
- 11 Strackharn, M., Stahl, S.W., Puchner, E.M., Gaub, H.E., 2012. *Nano Lett.* 12, 2425–2428.
- 12 Tombelli, S., Minunni, M., Mascini, M., 2005. *Biosens. Bioelectron.* 20, 2424-2434.
- 13 Ueno, S., Ono, A., Tanaka, Y., Sato, S., Biyani, M., Ichiki, T., 2012. *J. Photopolym. Sci. Technol.* 25, 67–72.
- 14 Wilson, D.S., Szostak, J.W., 1999. *Annu. Rev. Biochem.* 68, 611-647.
- 15 Xia, Y., Whitesides, G.M., 1998. *Angew. Chem. Int. Ed.* 37, 550-575.
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Figure legends

Figure 1. Schematic of temperature-controlled microintaglio printing (TC-μIP).

RNA solution is first applied onto a printing mold and the mold is pressed with a single-strand DNA (ssDNA)-modified gold-coated glass substrate at a low temperature T_1 . At a low temperature, the RNA is folded and hybridization is suppressed. After complete press-sealing of the mold and substrate, the temperature is increased to T_2 to denature the RNA, and the denatured RNA is hybridized with the ssDNA by gradual cooling to room temperature (RT). Finally, the glass substrate is released from the mold and the RNA is printed on the substrate. The temperatures T_1 and T_2 and incubation time t are controlled to fabricate a high-contrast RNA micropattern.

Figure 2. Effect of temperature T_1 during the press step.

(a) Temperature profile of the experiment. The press step was performed at $T_1 = 70, 25$, or 4°C for 5 min and $T_2 = 70^\circ\text{C}$ for 5 min, which was followed by gradual cooling to $\text{RT} = 25^\circ\text{C}$ at a rate of $0.1^\circ\text{C}/\text{s}$ and incubation for 10 min. (b) Result of μIP for each T_1 . The signal-to-background (S/B) ratios of the fluorescence signals of the printed Cy3-labeled RNA spots were 2.1, 9.5, and 12.5 for $T_1 = 70^\circ\text{C}$, 30°C , and 4°C , respectively. The S/B ratios were calculated from the line intensity between the white arrows in the figure.

Figure 3. Effect of denaturing time t during the press step.

(a) Temperature profile of the experiment. The press step was performed with $T_1 = 4^\circ\text{C}$, $T_2 = 70^\circ\text{C}$, and $t = 1$ -120 min, which was followed by gradual cooling to $\text{RT} = 25^\circ\text{C}$ at a rate of $0.1^\circ\text{C}/\text{s}$ and incubation for 10 min. (b) Result of μIP at each t . The fluorescence intensities of the printed spots of Cy3-labeled RNA fabricated with each incubation time t were measured. Error bars represent mean $\pm$ SD ($n = 9$ spots). Plots were fitted with a simple second-order kinetics curve.

Figure 4. Results of TC-μIP of RNA for several spot diameters.

(a) Fluorescence intensities per pixel of Cy5-labeled RNA spots with several diameters. The intensities were comparable among diameters of 4-50 μm. Error bars represent mean ± SD (n = 9-24 spots). (b) Downsized Cy5-labeled RNA spots with a diameter of 1.5 μm and a density of 40,000 spots/mm² obtained by TC-μIP.

Figure 5. Results of TC-μIP of RNA with arbitrary design.

A TC-μIP pattern of the letters “RNA” with a line width of 7.5 μm was fabricated using Cy5-labeled RNA molecules.

Figure 6. Microintaglio printing of *in situ* synthesized RNA.

(a) Schematic of TC-μIP based on *in situ* RNA synthesis. Microwells on a printing mold were filled with dsDNA-immobilized magnetic beads and an *in vitro* transcription reagent, and press-sealed with a capture-DNA-immobilized gold substrate. RNA molecules were *in situ* synthesized within each isolated well and then printed on the substrate via hybridization. *In situ* synthesis and hybridization were controlled via the temperature. (b) Results of TC-μIP based on *in situ* RNA synthesis. Upper: Bright-field microscopic image of the printing mold with bead-containing microwells. The beads were observed as dark spots and the empty wells were observed as gray spots. Lower: Fluorescence microscopic image of the substrate after TC-μIP. *In situ* synthesized Cy5-labeled RNA was printed on only wells containing DNA-immobilized beads.

Figure 1

Figure 1. Schematic of temperature-controlled microintaglio printing (TC-μIP)

As a first, RNA solution is applied onto the printing mold and the mold is pressed with a single-strand DNA (ssDNA)-modified gold-coated glass substrate at low temperature T_1 . At low temperature, it is thought that RNA is folded and hybridization was suppressed. After complete press-sealing of the mold and substrate, temperature is increase to T_2 to denature the RNA and the denatured RNA is hybridized with the ssDNA by gradual cooling to room temperature (RT). Finally, the glass substrate is released from the mold and RNA is printed on the substrate. Temperatures T_1 and T_2 , and incubation time t were controlled to fabricate a high-contrast RNA micropattern.

90 mm

Figure 2

Figure 2. Effect of temperature T_1 during the press step.

- (a) Temperature profile of the experiment. The press step was performed at $T_1 = 70, 25$, and 4°C for 5 min, $T_2 = 70^{\circ}\text{C}$ for 5 min, and followed by gradual cooling to $\text{RT} = 25^{\circ}\text{C}$ with ramp rate of 0.1°C/s and incubated for 10 min.
- (b) Result of μIP at each T_1 . Signal to background (S/B) ratios of fluorescence signal of printed Cy3-labeled RNA spots were 2.1, 9.5, and 12.5 at 70°C , 30°C , and 4°C , respectively. S/B ratios were calculated from the line intensity between white arrows.

90 mm

(a)

(b)

Figure 3

Figure 3. Effect of denaturing time t during the press step.

(a) Temperature profile of the experiment. The press step was performed at $T_1 = 4^\circ\text{C}$, $T_2 = 70^\circ\text{C}$, $t = 1\text{-}120$ min, and followed by gradual cooling to $\text{RT} = 25^\circ\text{C}$ with ramp rate of $0.1^\circ\text{C}/\text{s}$ and incubated for 10 min. Error bars represent mean $\pm$ SD ($n = 9$ spots). Plots were fitted with simple second-order kinetics curve.

(b) Result of μIP at each t . Fluorescence intensity of printed spots of Cy3-labeled RNA fabricated by each incubation time t were measured.

90 mm

Figure 4. Results of TC- μ IP of RNA for several diameters of spot.

(a) Fluorescence intensities per pixel of Cy5-labeled RNA spots with several diameters. The intensities were comparable among diameters of 4-50 μm . Error bars represent mean $\pm$ SD ($n = 9$ -24 spots).

(b) Downsized Cy5-labeled RNA spots with a diameter of 1.5 μm in a density of 40,000 spots/ mm^2 by TC- μ IP.

90 mm

Figure 5

Figure 5. Results of TC-μIP of RNA with arbitrary design.

TC-μIP pattern of character of “RNA” with line width of 7.5 μm was fabricated using Cy5-labeled RNA molecules.

90 mm

Figure 6

Figure 6. Microintaglio printing of *in situ* synthesized RNA.

(a) Schematic of TC-μIP based on *in situ* RNA synthesis. Microwells on the printing mold were filled with dsDNA-immobilized magnetic beads and *in vitro* transcription reagent, and press-sealed with the capture DNA-immobilized gold substrate. RNA molecule were *in situ* synthesized within each isolated well and the RNA molecules were printed on the substrate via hybridization. *In situ* synthesis and hybridization were controlled by temperature.

(b) Results of TC-μIP based on *in situ* RNA synthesis. Upper: Bright-field microscopic image of the beads-containing microwell printing mold. The beads were observed as black spots and empty wells were observed as white. Bottom: Fluorescence microscopic image of the substrate after TC-μIP. *In situ* synthesized Cy5-labeled RNA was printed on only wells containing DNA-immobilized beads.