

Toehold-initiated Rolling Circle Amplification for Visualizing Individual MicroRNAs In Situ in Single Cells**

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Abstract: The ability to quantitate and visualize microRNAs (miRNAs) in situ in single cells would greatly facilitate the elucidation of miRNA-mediated regulatory circuits and their disease associations. A toehold-initiated strand-displacement process was used to initiate rolling circle amplification of specific miRNAs, an approach that achieves both stringent recognition and in situ amplification of the target miRNA. This assay, termed toehold-initiated rolling circle amplification (TIRCA), can be utilized to identify miRNAs at physiological temperature with high specificity and to visualize individual miRNAs in situ in single cells within 3 h. TIRCA is a competitive candidate technique for in situ miRNA imaging and may help us to understand the role of miRNAs in cellular processes and human diseases in more detail.

MicroRNAs (miRNAs) are crucial regulators of gene expression.^[1] The abnormal expression of miRNAs may contribute to serious diseases, such as cancers,^[2] cardiovascular disease,^[3] and neurological disorders.^[4] Given the considerable cell-to-cell variation in miRNA expression, the ability to visualize miRNAs in single cells, particularly with single-molecule resolution, is essential for resolving miRNA-mediated regulatory circuits and the complexity and heterogeneity of miRNA-related diseases. However, visualizing intracellular miRNAs is challenging because of their small size, highly homologous sequences, and sometimes low-level expression. Currently, the most common method for visualizing gene expression inside cells is fluorescence in situ hybridization (FISH). Single-molecule transcripts can be detected in situ by FISH by using multiple fluorescently labeled oligonucleotide probes.^[5] However, shortening the probes greatly reduces their mismatch discrimination and thus limits their use for identifying short miRNAs (approximately 18–25 nucleotides (nt) long) with only minor sequence differences. Moreover, only one probe can be hybridized to the short target miRNA, thereby yielding inadequate fluorescence to quantify the miRNA expression in individual cells, especially in cells with low miRNA expres-

sion.^[6] Stringent recognition and in situ amplification strategies are thus imperative for intracellular miRNA imaging.

Toehold-mediated strand displacement (TMSD) is a controllable nonenzymatic process in which one strand of DNA in a double-stranded complex is displaced by another DNA strand with the help of a short single-stranded neighboring sequence (called the toehold).^[7] Owing to its predictable thermodynamics and kinetics, TMSD has been widely used in DNA nanotechnology, for example, in DNA circuits^[8] and DNA nanomachines.^[9] Remarkably, TMSD has been demonstrated to boost the specificity of nucleic-acid recognition compared to simple Watson–Crick base pairing.^[10] Robust sequence discrimination was achieved across diverse temperature, salt, and concentration conditions by using ‘toehold exchange’ probes,^[11] thus indicating the feasibility of using TMSD for identifying sequences in complex environments such as inside cells or in peripheral blood. Initial investigations have utilized TMSD to quantify highly homologous sequences in vitro^[12] and even to image single-nucleotide polymorphisms (SNPs) in situ in single cells.^[13] Despite this progress, the low signal output of TMSD remains an obstacle to applying this technique to the visualization of short miRNAs. Typical TMSD is a toehold-mediated single-strand release process, which cannot provide sufficient signal (in most cases, fluorescence) for locating miRNAs inside cells.

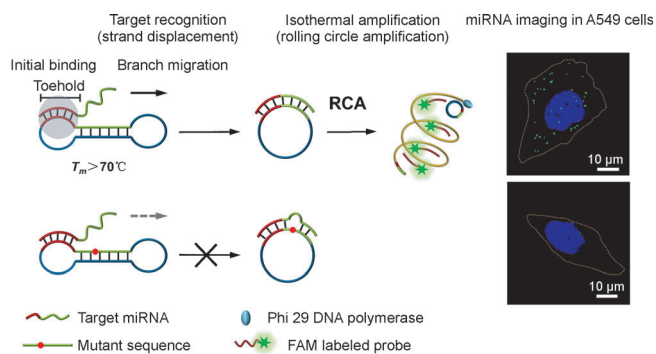
Instead of releasing a single strand by TMSD, we utilized TMSD to initiate rolling circle amplification (RCA) by constructing a structure-switchable dumbbell-shaped probe (called a seal probe). The toehold-initiated RCA method, termed TIRCA, is a potent strategy that enables both stringent recognition and in situ amplification of the target sequences. We have found that TIRCA can be utilized to detect miRNAs with much higher specificity than RCA or padlock-probe-based RCA and even to realize the visualization of individual miRNAs in situ in single cells.

Our strategy is illustrated in Scheme 1. Unlike typical TMSD, which uses a toehold-bearing linear DNA duplex, TIRCA makes use of a dumbbell-shaped seal probe with a tunable toehold on its loop. The seal probe acts as both the probe for TMSD and the template for the subsequent RCA. Initially, the seal probe remains stable as a dumbbell-shaped structure, which is ‘closed’ for RCA. When the target miRNA binds to the toehold domain, spontaneous branch migration results in a switch to the ‘activated’ circular form, thereby initiating RCA. However, a mismatched miRNA will fail to migrate through because of the resistance of the stabilized dumbbell structure, thus yielding no amplification. After opening up the seal probe, the target miRNA is extended to hundreds of tandem repeats by RCA. Through hybridization with a FAM-labeled probe, a fine diffraction-limited spot

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Scheme 1. Schematic representation of TIRCA for visualizing individual miRNAs in situ inside cells. After binding to the toehold domain of the seal probe, the target miRNA switches the seal probe to the 'activated' circular form through a strand-displacement process, thereby initiating RCA. When there is a sequence mismatch between the miRNA and the seal probe, the miRNA fails to branch migrate through the probe and there is no amplification. The hybridization of the RCA product (RCAP) with 6-carboxyfluorescein (FAM)-labeled probes leads to a bright spot for each target miRNA molecule recognized. TIRCA was applied to visualize individual miRNAs in A549 cells. RCAPs are shown as green dots in an A549 cell with its nucleus stained by 4',6-diamidino-2-phenylindole (DAPI; blue), and the outline of A549 cell is marked with a dotted line

corresponding to single miRNA can be distinguished from the background. High specificity for miRNA detection inside the lung cancer cell line A549 cells has been achieved. Abundant bright spots emerged inside A549 cells when using perfectly matched seal probes for the miRNA let-7a. By contrast, only rare bright spots could be seen when there was a one-base mismatch between the seal probe and let-7a.

Given that TMSD is a toehold-dependent process, the effects of toehold length on the kinetics of TIRCA were investigated. The TIRCA products can form large amounts of ssDNA and dsDNA (Figure 1A), which can be detected by using the intercalating dye Sybr Green I. The TIRCA process was monitored through real-time fluorescence measurements. The time taken to reach half of the maximum fluorescence ($C_{T1/2}$) was used as a quantitative kinetic parameter for RCA.^[14] When the toehold length was increased from 6 to 10 nt, $C_{T1/2}$ decreased from 62 to 23 min (Figure 1C), thus indicating that a longer toehold facilitates TIRCA. Nevertheless, when the toehold length was increased above 9 nt, $C_{T1/2}$ changed only slightly (a reduction of 5 min), thus suggesting that the TMSD process occurs rapidly with a long toehold (> 9 nt) and is no longer the rate-limiting step for TIRCA. TIRCA is a multistep reaction comprising two processes: TMSD and RCA. The $C_{T1/2}$ value for RCA when using a circular probe was measured to be 9 min. After introducing the TMSD process by using the seal probe, $C_{T1/2}$ increased by 14–53 min (with toeholds ranging from 10 nt down to 6 nt), thus indicating that TMSD plays a significant role in TIRCA and that the toehold length has a substantial effect on the rate of TIRCA. The decrease in the fluorescence intensity in the later stages of TIRCA (Figure 1B) may result from the precipitation of the large TIRCA products, which have complex secondary structure.^[15]

To investigate whether the TMSD process could effectively improve the specificity of miRNA detection, let-7

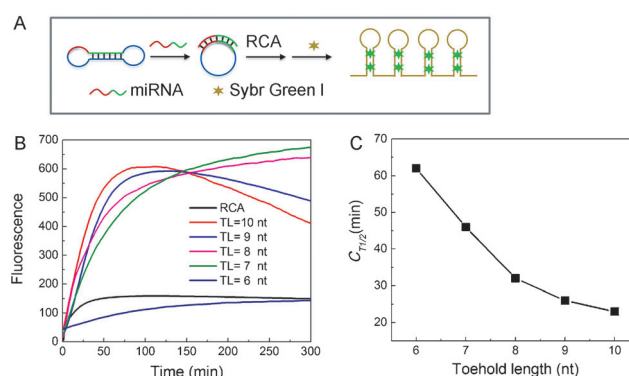


Figure 1. The effect of toehold length (TL) on the kinetics of TIRCA. A) Scheme for miRNA detection by TIRCA in vitro. B) Real-time fluorescence measurements for RCA (with a circular probe) and TIRCA (with seal probes with different TLs). The concentration of let-7a was 1 nM. The excitation wavelength was 494 nm and the emission wavelength was 521 nm. C) Plots of $C_{T1/2}$ against TL for TIRCA. The relatively low fluorescence intensity when using a seal probe with a 6-nt toehold is a result of lower amplification efficiency.

family members (let-7a–g) with high sequence homology were chosen to be detected by TIRCA (Figure 2, Figure S4 in the Supporting Information). Let-7 miRNAs function as tumor suppressors in lung cells and their reduced expression in human lung cancers is associated with shortened post-operative survival.^[16] Circular ssDNA is typically used as the template for standard RCA, in which target recognition is based on simple Watson–Crick base pairing between the target and the circular probe. The fluorescence intensity change compared to the background (ΔF) produced by let-7a was only 1.14-fold higher than that produced by let-7f (one-base mismatched) when using the circular probe for let-7a (Figure 2B), thus indicating that standard RCA cannot resolve the let-7 family members. Padlock probes, which can be circularized by T4 DNA ligase upon perfect hybridization with the target sequence, are used to improve the specificity of RCA, and padlock-probe-based RCA (PRCA) has been successfully applied to detect SNPs.^[17] However, owing to the reduced specificity of the ligase on an RNA template,^[18] PRCA showed limited improvement to the specificity of miRNA detection. The ΔF value produced by let-7a was only 2.77-fold higher than that produced for let-7f. Dumbbell-shaped seal probes enable the utilization of TMSD to identify target miRNA. The ΔF value for let-7a was 22.20-fold higher than that of let-7f when using a seal probe with a 7-nt toehold, a much larger difference than when using RCA or PRCA. Even if the mismatched base was at the 3' terminus (for example, in let-7c), the discrimination factor was still up to 11.15. TMSD can thus effectively improve the specificity of miRNA detection and TIRCA possesses sufficiently high specificity to resolve miRNA family members with single-nucleotide variations. The detection limit of TIRCA could be as low as 0.72 fM (3 times the standard deviation of background; Figure S2); TIRCA is thus a highly sensitive and selective strategy for quantifying miRNA in vitro.

Next, we investigated the mechanism of the enhanced specificity of TIRCA for miRNA detection. Seal probes with different toehold lengths were constructed. All of these seal

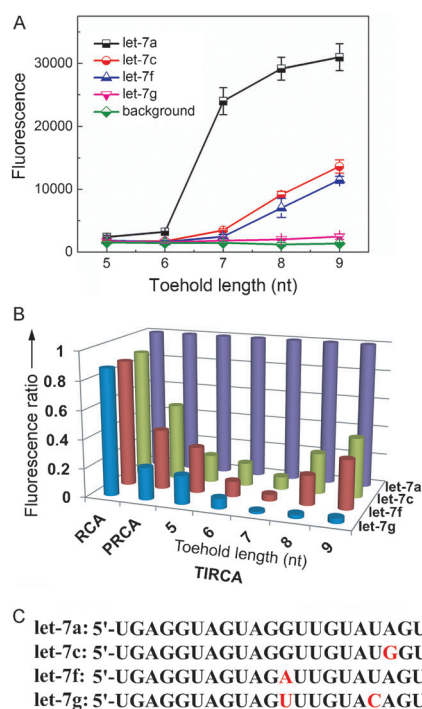


Figure 2. The effect of the toehold length on the specificity of TIRCA. TIRCA was carried out at 1 nM let-7a (target miRNA), let-7f (single mismatched), let-7c (single mismatched), or let-7g (double mismatched) with seal probes with different toehold lengths. A) Plots of fluorescence intensity at 520 nm against toehold length. The excitation wavelength was 494 nm. Error bars were obtained from three parallel experiments. B) The fluorescence ratios $\Delta F_a/\Delta F_b$ (let-7a), $\Delta F_a/\Delta F_c$ (let-7c), $\Delta F_a/\Delta F_f$ (let-7f), and $\Delta F_a/\Delta F_g$ (let-7g) for RCA, PRCA, and TIRCA with seal probes with different toehold lengths. $\Delta F_{a,c,f,g}$ are the fluorescence intensity changes compared to background fluorescence for let-7a, c, f, g, respectively, and the concentration of miRNA was 1 nM in each case. C) The sequences of let-7a, let-7c, let-7f, and let-7g. The bases that differ from those in let-7a are marked in red.

probes were highly stable; the T_m values for the seal probes with 5–9-nt toeholds ranged from 81.5 to 74.5 °C (Table S2 in the Supporting Information). Initially, the self-complementary seal probe remains a dumbbell-shaped structure, a closed state that cannot act as the template for RCA. Only target hybridization with a larger thermodynamic energy change than that of the opening process of the seal probe can switch the seal probe to the ‘activated’ circular form. For a circular probe without secondary structure, nonspecific hybridization with one or more mismatches may still initiate RCA, thereby resulting in low specificity. The stability of the seal probes is greatly dependent on the toehold length and is a key factor in the specificity of miRNA detection by TIRCA. The highest discrimination factor was achieved by using a seal probe with 7-nt toehold. When the toehold length was greater than 7-nt, the seal probe was not stable enough to resist strand migration of the single mismatched miRNAs let-7c and let-7f, and as a result, the TIRCA assay exhibited lower selectivity. On the other hand, when the toehold length was smaller than 7 nt, the seal probe was so stable that even the target miRNA could not open the probe to initiate RCA; as a result, the fluorescence intensity for all of the miRNAs

decreased to close to background. The optimal toe-hold length will confer a degree of stability on the self-complementary state of the seal probe that is sufficiently high to resist nonspecific hybridization but not so high as to prevent strand migration of the target miRNA.

The standard free energy change (ΔG) for TMSD was calculated to further explain the effects of toehold length on the specificity of TIRCA. The ΔG value for TMSD increases when the stability of the seal probe is improved by shortening its toehold. Mismatched hybridization between the miRNAs (let-7f, c, and g) and the seal probe also raises ΔG . TIRCA barely occurred with positive ΔG values and was favored by lower ΔG values (Table S4). When using the seal probe with a 7-nt toehold, the ΔG value for let-7a was $-2.93 \text{ kJ mol}^{-1}$, whereas the ΔG values for let-7c and let-7f were 4.18 kJ mol^{-1} and $16.74 \text{ kJ mol}^{-1}$, respectively. Nonspecific amplification was thus effectively suppressed and the highest discrimination factor was achieved. The key principle in designing seal probes is achieving TMSD with large positive values of ΔG for the mismatched miRNA and negative values of ΔG for the target miRNA. High specificity was also achieved when using seal probes designed for let-7d and let-7g (Table S5, and Figure S5). Although the values from the thermodynamic calculations may deviate from the experimental values because TMSD involves complex DNA structures, these findings may still be helpful for the preliminary design of seal probes.

After verification and optimization in vitro, we explored the potential of TIRCA for visualizing miRNA in situ in the lung cancer cell line A549 cells. In situ TIRCA was conducted with optimized seal probes at 37 °C for 90 min. All the in situ imaging processes were performed within 3 h. RCA is a localized isothermal amplification, which means that it can provide information about the localization of the target molecules with single-molecule resolution.^[17b,19] Single miRNA molecules were extended to long chains containing hundreds of tandem repeats and became visible as diffraction-limited fluorescent spots upon hybridization to fluorescent probes.^[17b] The abundant fluorescence-enhanced bright spots could be visualized by using confocal microscopy (Figure 3). Larger signals of variable morphology may correspond to several adjacent RCA products (RCAPs).^[19a] Fluorescence images revealed that individual bright fluorescent spots, which presumably corresponded to individual miRNAs, were mostly located randomly in cytoplasm with no obvious aggregation seen (Figure 3B). The number of RCAPs varied considerably between cells, with counts ranging from 14 to 93 per cell (the average was 39.88 per cell; inset in Figure 3B). This variability indicates that miRNA expression can be distinct even in cells of the same type because the individual cells may be at different stages.

Next, FISH was performed as a comparison. The A549 cells were fixed and hybridized in situ with a FAM-labeled FISH probe against let-7a, following by thorough washing. No fluorescence signal above background was observed (more than 100 cells checked), thus FISH failed to detect the let-7a that is reported to be expressed at low levels in A549 cells.^[16b] Additionally, to confirm that the signal resulted from the target miRNA let-7a, we blocked let-7a with an unlabeled

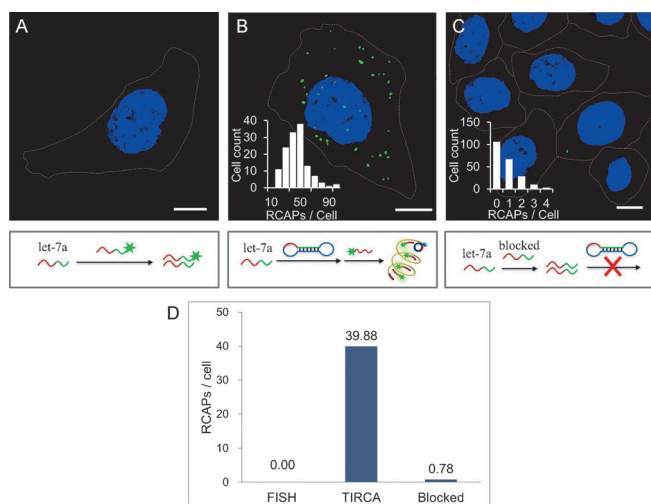


Figure 3. Evaluation of TIRCA for target miRNA imaging in A549 cells. A) Let-7a imaging by FISH. B) Let-7a imaging by TIRCA. C) TIRCA after let-7a was blocked. Scale bars: 10 μ m. The cell nuclei are shown in blue, the RCAPs appear as green spots, and the cell outlines are marked by a dotted line. Insets: Frequency histogram of RCAPs in the cells. D) Quantification of the average number of RCAPs per cell detected by FISH (126 cells counted), TIRCA (132 cells counted), and TIRCA after let-7a was blocked (213 cells counted).

probe complementary to let-7a before carrying out TIRCA. Only less than one RCAP per cell could be seen (Figure 3D), thus suggesting that the bright spots came from the target miRNA let-7a. We estimated the in situ detection efficiency of TIRCA to be 30–45 % (32.26 % for let-7a, 41.10 % for let-7d) on the basis of a comparison to quantitative PCR (qPCR) data (Table S6). TIRCA measurements of the relative levels of let-7a and let-7d in A549 cells showed good correlation with the qPCR results, and both techniques produced values in good agreement with the literature.^[20]

TMSD has been successfully used to improve the specificity of TIRCA in vitro. To test the specificity of TIRCA inside cells, the seal probes used for the imaging of let-7a and let-7d were altered by one or two bases (Table S1). The number of RCAPs per cell decreased sharply after the introduction of a one- or two-base mismatch (Figure 4 and Figure S7). The average numbers of RCAPs per cell when using one-base mismatched (Mis-1) and two-base mismatched (Mis-2) seal probes for let-7a were 1.12 per cell and 0.25 per cell respectively, much less than the 44.44 RCAPs per cell measured with the matched probe (Figure 4D). The discrimination factor between a perfect match and a one-base mismatch was estimated up to be 39.68 for let-7a and 35.20 for let-7d. This high TIRCA specificity was achieved at physiological temperature (37 °C), whereas FISH with locked nucleic acid (LNA) conferred selectivity for miRNA detection only at much higher temperatures (above 50 °C),^[21] which may cause the miRNA to leak from the cells.^[6b]

In summary, we applied toehold-mediated strand displacement (TMSD) to initiate the rolling circle amplification of specific RNAs by engineering a structure-switchable seal probe. This miRNA assay, termed toehold-initiated rolling circle amplification (TIRCA), achieves both stringent recog-

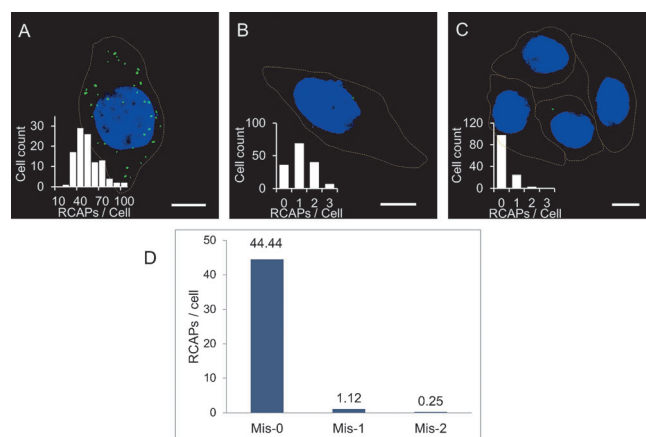


Figure 4. Demonstration of the specificity of TIRCA for miRNA imaging in A549 cells. In situ detection of let-7a in A549 cells was performed by using perfectly matched (Mis-0; A), single mismatched (Mis-1; B), and double mismatched (Mis-2; C) seal probes. Scale bars: 10 μ m. The cell nuclei are shown in blue, the RCAPs appear as green spots, and the cell outlines are marked by a dotted line. Insets: Frequency histogram of RCAPs in the cells. D) Quantification of the average number of RCAPs detected in A549 cells by using Mis-0 (105 cells counted), Mis-1 (152 cells counted), Mis-2 (126 cells counted) seal probes.

nition and in situ amplification of the target miRNA and allows the visualization of individual miRNAs in single cells. TIRCA has many advantages: 1) by taking advantage of the fine sequence discrimination of TMSD, highly specific enzyme-independent recognition of miRNAs is achieved; 2) as little as a single molecule of miRNA can be visualized following the localized amplification; 3) with the help of a seal probe, the recognition and amplification processes are realized in one step, which greatly shortens the time needed for miRNA imaging; and 4) all of miRNA-imaging steps proceed at physiological temperature, thus reducing the loss of miRNAs from the cells. TIRCA is thus a competitive candidate technique for the identification and in situ imaging of miRNAs and may help us to understand the role of miRNAs in cellular processes and human diseases in more detail. Additionally, this work could inspire the integration of TMSD into various nucleic-acid amplification techniques to build diverse selective assays for detecting low-concentration nucleic acids (or proteins, or small biomolecules) in complex real-world samples.

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- [1] a) M. Selbach, B. Schwanhäusser, N. Thierfelder, Z. Fang, R. Khanin, N. Rajewsky, *Nature* **2008**, 455, 58–63; b) L. He, G. J. Hannon, *Nat. Rev. Genet.* **2004**, 5, 522–531; c) D. P. Bartel, *Cell* **2009**, 136, 215–233.

- [2] a) A. Lujambio, S. W. Lowe, *Nature* **2012**, *482*, 347–355; b) G. A. Calin, C. M. Croce, *Nat. Rev. Genet.* **2006**, *7*, 857–866; c) A. Esquela-Kerscher, F. J. Slack, *Nat. Rev. Genet.* **2006**, *7*, 259–269.
- [3] a) E. M. Small, E. N. Olson, *Nature* **2011**, *469*, 336–342; b) T. Thum, C. Gross, J. Fiedler, T. Fischer, S. Kissler, M. Bussen, P. Galuppo, S. Just, W. Rottbauer, S. Frantz, *Nature* **2008**, *456*, 980–984.
- [4] a) S. Haramati, E. Chapnik, Y. Sztainberg, R. Eilam, R. Zwang, N. Gershoni, E. McGlinn, P. W. Heiser, A. M. Wills, I. Wirguin, *Proc. Natl. Acad. Sci. USA* **2010**, *107*, 13111–13116; b) S. M. Eacker, T. M. Dawson, V. L. Dawson, *Nat. Rev. Neurosci.* **2009**, *10*, 837–841.
- [5] a) S. Itzkovitz, A. Van Oudenaarden, *Nat. Methods* **2011**, *8*, S12–S19; b) Y. Taniguchi, P. J. Choi, G. W. Li, H. Chen, M. Babu, J. Hearn, A. Emili, X. S. Xie, *Science* **2010**, *329*, 533–538; c) A. M. Femino, F. S. Fay, K. Fogarty, R. H. Singer, *Science* **1998**, *280*, 585–590; d) D. Zenklusen, D. R. Larson, R. H. Singer, *Nat. Struct. Mol. Biol.* **2008**, *15*, 1263–1271.
- [6] a) R. Thomsen, P. S. Nielsen, T. H. Jensen, *RNA* **2005**, *11*, 1745–1748; b) J. T. Pena, C. Sohn-Lee, S. H. Rouhanifard, J. Ludwig, M. Hafner, A. Mihailovic, C. Lim, D. Holoch, P. Berninger, M. Zavolan, *Nat. Methods* **2009**, *6*, 139–141.
- [7] a) D. Y. Zhang, G. Seelig, *Nat. Chem.* **2011**, *3*, 103–113; b) D. Y. Zhang, E. Winfree, *J. Am. Chem. Soc.* **2009**, *131*, 17303–17314; c) Y. Krishnan, F. C. Simmel, *Angew. Chem.* **2011**, *123*, 3180–3215; *Angew. Chem. Int. Ed.* **2011**, *50*, 3124–3156.
- [8] a) G. Seelig, D. Soloveichik, D. Y. Zhang, E. Winfree, *Science* **2006**, *314*, 1585–1588; b) B. M. Frezza, S. L. Cockcroft, M. R. Ghadiri, *J. Am. Chem. Soc.* **2007**, *129*, 14875–14879; c) L. Qian, E. Winfree, *Science* **2011**, *332*, 1196–1201.
- [9] a) J. Bath, A. J. Turberfield, *Nat. Nanotechnol.* **2007**, *2*, 275–284; b) B. Yurke, A. J. Turberfield, A. P. Mills, F. C. Simmel, J. L. Neumann, *Nature* **2000**, *406*, 605–608; c) P. Yin, H. M. Choi, C. R. Calvert, N. A. Pierce, *Nature* **2008**, *451*, 318–322.
- [10] Q. Li, G. Luan, Q. Guo, J. Liang, *Nucleic Acids Res.* **2002**, *30*, e5.
- [11] D. Y. Zhang, S. X. Chen, P. Yin, *Nat. Chem.* **2012**, *4*, 208–214.
- [12] a) H. K. Subramanian, B. Chakraborty, R. Sha, N. C. Seeman, *Nano Lett.* **2011**, *11*, 910–913; b) D. Wang, W. Tang, X. Wu, X. Wang, G. Chen, Q. Chen, N. Li, F. Liu, *Anal. Chem.* **2012**, *84*, 7008–7014; c) Z. Zhang, D. Zeng, H. Ma, G. Feng, J. Hu, L. He, C. Li, C. Fan, *Small* **2010**, *6*, 1854–1858.
- [13] M. J. Levesque, P. Ginart, Y. Wei, A. Raj, *Nat. Methods* **2013**, *10*, 865–867.
- [14] J. Pickering, A. Bamford, V. Godbole, J. Briggs, G. Scozzafava, P. Roe, C. Wheeler, F. Ghouze, S. Cuss, *Nucleic Acids Res.* **2002**, *30*, e60.
- [15] Y. Tian, Y. He, C. Mao, *ChemBioChem* **2006**, *7*, 1862–1864.
- [16] a) C. D. Johnson, A. Esquela-Kerscher, G. Stefani, M. Byrom, K. Kelnar, D. Ovcharenko, M. Wilson, X. Wang, J. Shelton, J. Shingara, *Cancer Res.* **2007**, *67*, 7713–7722; b) J. Takamizawa, H. Konishi, K. Yanagisawa, S. Tomida, H. Osada, H. Endoh, T. Harano, Y. Yatabe, M. Nagino, Y. Nimura, *Cancer Res.* **2004**, *64*, 3753–3756.
- [17] a) X. Qi, S. Bakht, K. M. Devos, M. D. Gale, A. Osbourn, *Nucleic Acids Res.* **2001**, *29*, e116; b) X. Zhong, P. M. Lizardi, X. Huang, P. Bray-Ward, D. Ward, *Proc. Natl. Acad. Sci. USA* **2001**, *98*, 3940–3945; c) J. Li, T. Deng, X. Chu, R. Yang, J. Jiang, G. Shen, R. Yu, *Anal. Chem.* **2010**, *82*, 2811–2816.
- [18] a) E. M. Harcourt, E. T. Kool, *Nucleic Acids Res.* **2012**, *40*, e65; b) M. Nilsson, G. Barbany, D. Antson, K. Gertow, U. Landegren, *Nat. Biotechnol.* **2000**, *18*, 791–793; c) M. Nilsson, D. O. Antson, G. Barbany, U. Landegren, *Nucleic Acids Res.* **2001**, *29*, 578–581; d) Y. Cheng, X. Zhang, Z. Li, X. Jiao, Y. Wang, Y. Zhang, *Angew. Chem.* **2009**, *121*, 3318–3322; *Angew. Chem. Int. Ed.* **2009**, *48*, 3268–3272.
- [19] a) C. Larsson, J. Koch, A. Nygren, G. Janssen, A. K. Raap, U. Landegren, M. Nilsson, *Nat. Methods* **2004**, *1*, 227–232; b) C. Larsson, I. Grundberg, O. Söderberg, M. Nilsson, *Nat. Methods* **2010**, *7*, 395–397; c) S. Huang, C. Yu, G. Cheng, Y. Chen, *Anal. Chem.* **2012**, *84*, 8118–8121; d) P. M. Lizardi, X. Huang, Z. Zhu, P. Ward, D. Thomas, D. Ward, *Nat. Genet.* **1998**, *19*, 225–232.
- [20] P. Landgraf, M. Rusu, R. Sheridan, A. Sewer, N. Iovino, A. Aravin, S. Pfeffer, A. Rice, A. O. Kamphorst, M. Landthaler, *Cell* **2007**, *129*, 1401–1414.
- [21] a) W. P. Kloosterman, E. Wienholds, E. de Bruijn, S. Kauppinen, R. H. Plasterk, *Nat. Methods* **2005**, *2*, 27–29; b) G. Obernosterer, J. Martinez, M. Alenius, *Nat. Protoc.* **2007**, *2*, 1508–1514; c) A. N. Silahatoglu, D. Nolting, L. Dyrskjöt, E. Berezikov, M. Møller, N. Tommerup, S. Kauppinen, *Nat. Protoc.* **2007**, *2*, 2520–2528; d) J. Lu, A. Tsourkas, *Nucleic Acids Res.* **2009**, *37*, e100.