Direct structural analysis of modified RNA by fluorescent in-line probing

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ABSTRACT

Chemical probing is a common method for the structural characterization of RNA. Typically, RNA is radioactively end-labelled, subjected to probing conditions, and the cleavage fragment pattern is analysed by gel electrophoresis. In recent years, many chemical modifications, like fluorophores, were introduced into RNA, but methods are lacking that detect the influence of the modification on the RNA structure with single-nucleotide resolution. Here, we first demonstrate that a 5'‑terminal $^{32}$P label can be replaced by a dye label for in-line probing of riboswitch RNAs. Next, we show that small, highly structured FRET-labelled Diels–Alderase ribozymes can be directly probed, using the internal or terminal FRET dyes as reporters. The probing patterns indeed reveal whether or not the attachment of the dyes influences the structure. The existence of two dye labels in typical FRET constructs is found to be beneficial, as ‘duplexing’ allows observation of the complete RNA on a single gel. Structural information can be derived from the probing gels by deconvolution of the superimposed band patterns. Finally, we use fluorescent in-line probing to experimentally validate the structural consequences of photocaging, unambiguously demonstrating the intentional destruction of selected elements of secondary or tertiary structure.

INTRODUCTION

Chemical and enzymatic probing of structured RNA species is of outstanding importance for RNA biochemistry (1–4). Usually, these techniques provide the first (and often only) insight into the structural basis of ribozyme function (5–8) or aptamer-target recognition (9–11). One of the simplest chemical probing techniques is in-line probing, where the RNA is incubated at slightly alkaline pH, and the spontaneous cleavage of the sugar backbone by adjacent 2'-hydroxyl groups is monitored. The cleavage rate of an individual phosphodiester group depends on the ionization state of the 2'-OH group, the distance between 2'-OH and phosphorus, and the correct in-line orientation of incoming nucleophile, electrophilic centre and leaving group (12). The latter two parameters are correlated to the local flexibility: in regular helical parts this in-line conformation cannot be formed, causing a lack of cleavage bands in these regions. In contrast, extended unstructured, ‘random coil’ regions have a high degree of conformational freedom so that in-line conformations are statistically adopted, leading to rather uniform ladder-like cleavage band patterns. Strong cleavage bands, however, will be detected when the phosphodiester bond is fixed in an optimal in-line conformation. In-line probing can be conducted in a titration-type manner to study binding interactions and thereby even allows mechanistic conclusions (13). Commonly, RNA is radioactively end-labelled for these studies, and the cleavage fragments are analysed by gel electrophoresis (14).

In past years, an increasing number of chemical modifications have been introduced into RNA and studied in a biological context. Fluorescently labelled RNA molecules were used in smFRET (single-molecule FRET) folding studies (15–19), ribozyme activity assays (20,21) or high-resolution microscopy (22). Furthermore, the molecular characteristics of RNAs were expanded and tuned by incorporating a multitude of different modifiers. One example are RNAs that have been specifically modified with photocleavable protection groups (caged RNAs) to unravel folding processes or to switch functional states of RNAs (23,24). However, it is often only assumed that the highly modified RNA preserves all structural characteristics of its unmodified parent. Yet, there is no suitable technique available to directly verify this assumption. A FRET efficiency value in the expected range can at most confirm the global fold of the RNA of interest, while local perturbations caused by the dyes or other modifiers are impossible to resolve. Such

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information, on the other hand, could be provided by chemical probing. Therefore, we asked whether the fluorescent labels already present in the RNA molecules could be utilized to gather structural information by in-line probing, without the need to incorporate additional radioactive labels, and without the disadvantages associated with radioactive work.

Here, we first demonstrate that a 5′-terminal 32P label can be directly replaced by a dye label for in-line probing of riboswitches. Side-by-side comparison shows near-identical probing patterns and binding constants of its cognate ligand. Next, we show that small, highly structured FRET-labelled Diels–Alderase (DAse) ribozymes can be directly probed, and the probing patterns reveal whether or not the attachment of the dyes influences the correct folding. Finally, we probe highly modified RNAs, carrying FRET dyes, photocaging groups and a biotin tag, that are not accessible to 5′- or 3′-32P labelling, and provide structural information about these molecules. The existence of two dye labels in typical FRET constructs is found to be beneficial, as additional structural information can be derived from the probing gels by deconvolution of the superimposed band patterns.

MATERIALS AND METHODS

Oligonucleotides

*Bacillus subtilis* was cultured overnight at 37°C, 250 rpm in LB-medium. Genomic DNA was isolated with the GenElute Bacterial Genomic DNA Kit (Sigma), and the 5′-UTR of the *lysC* gene from 305 to 126 nt before the start of the coding region was amplified by PCR using the forward primer (additionally containing a T7-promoter sequence): 5′-TAA TAC GAC TCA TAG GTG AAG ATA GAG GTG CG-3′, and the reverse primer: 5′-AAC GAG ATC GCC CTC CAA GA-3′, applying the supplier’s standard protocol with an annealing temperature of 57°C using *Taq* polymerase. The product obtained was transcribed with T7 RNA polymerase to yield the 179 nt riboswitch aptamer. A total of 600 pmol of this product were dephosphorylated, thiophosphorylated and fluorescein labelled by deconvolution of the superimposed band patterns.

A two-way RNA ligation system was used to assemble the constructs A–D (3′-27-mer+23-mer-5′), whereas a three-way RNA ligation system was used to assemble the NPE-protected 49-mer constructs E–H (3′-14-mer+13-mer+22-mer-5′) essentially as described previously (28). All ligation products were purified on preparative gels (denaturing PAGE, 12.5%). The high purity of all DAse constructs was verified by analysing the samples on a denaturing sequencing gel (PAGE, 15%) and scanning for Cy3 and Cy5 fluorescence with a Typhoon 9400 gel scanner (GE Healthcare).

In-line probing

*Lysine Riboswitch Aptamer*: 6 pmol of the fluorescein labelled riboswitch or 0.06 pmol of 5′-32P-labelled riboswitch (29) were dissolved in a volume of 20 μl containing 50 mM Tris–HCl buffer (pH 8.5 at 25°C), 100 mM KCl and 20 mM MgCl2, in the presence of 20 μg *Escherichia coli* total tRNA. If indicated, the samples were supplemented with 1 mM to 1 mM L-lysine (Sigma). After
unfolding for 4 min at 70°C, the RNA was refolded by controlled cooling to 4°C at a rate of 0.2 K/s, and left at that temperature for 10 min. Then the probing reaction was performed at 35°C for 24 h until it was quenched by addition of gel loading buffer containing 7 M urea, 10 mM EDTA and bromophenol blue dye.

Partial alkaline digest was done by incubation of 6 pmol fluorescently labelled RNA or 0.06 pmol of 5′-32P-labelled riboswitch in 10 μl 50 mM NaHCO3 buffer (pH 9) with 10 μg E. coli total tRNA at 90°C for 5 min and subsequent immediate quenching of the reaction on ice and addition of gel loading buffer.

G-specific sequencing ladders were generated by incubation of 6 pmol fluorescently labelled RNA or 0.06 pmol of 5′-32P-labelled riboswitch with 10 μg E. coli total tRNA in 10 μl 12.5 mM sodium citrate buffer (pH 4.5) containing 3.5 M urea and 0.5 mM EDTA. After denaturation of the RNA for 10 min at 55°C, 0.4 U (2 μl) RNase T1 were added and the sample incubated for additional 10 min at 55°C. Then the reaction was terminated by addition of gel loading buffer.

The cleavage products were separated by loading 6 μl samples on 10% PAGE gels. Gels were run for 3 h at 45 W and visualized by scanning the Atto 633 label fluorescence at 532 nm. Image processing was performed using ImageJ software (31). Subtraction images were obtained by background subtraction in both scans and subsequent normalization. Images derived from the terminal label were then subtracted from the respective internal counterpart and all intensity values <0 were set to zero.

RESULTS
Replacement of a terminal 32P label in simple probing experiments

In order to test whether a terminal 32P label could be replaced by a fluorescent dye, we performed in-line probing on the 179-mer riboswitch aptamer from the lysC gene of B. subtilis, which is the second-largest known natural riboswitch motif. As the lysine riboswitch is generally preformed, only those nucleotides in the binding pocket that actively take part in the recognition of the ligand or that are in close proximity change their cleavage intensity in an in-line probing experiment. Determination of these slight changes in a long RNA is more demanding than the detection of global refolding events observed in other riboswitches.

Riboswitch RNA was generated by in vitro transcription and 5′-labelled with Atto 633 or 32P. Both RNAs were subjected to in-line probing with varying concentrations of L-lysine in the probing buffer (1 mM to 1 M). Both labels were visualized on the same instrument (Typhoon 9400 Gel Scanner). From the resulting cleavage patterns the apparent dissociation constants were derived (Figure 1a and b).

The data obtained with both methods are in good agreement with each other and with earlier reports (32). The overall band patterns are stable and reproducible with the fluorescent approach. Three regions of attenuated cleavage can be observed with increasing lysine concentrations, as they participate in binding the metabolite. While nucleotides 84–86 (Region 3, Figure 1c) are known to form contacts to the ω-amine of the lysine side chain, nucleotides 148–149 (Region 2) hydrogen-bond to the acid functionality and the ω-amine. Upon binding, the pocket obtains further stabilization, which can be seen from the reduced cleavage of nucleotides 170–174 (Region 1) (33,34).
The intensity of the cleavage bands observed in all three regions corresponds very well with the values obtained with $^{32}$P (Figure 1d). Figure 1e shows the determination of the apparent dissociation binding constant using both methods. For the fluorescent label, the $K_d$ was determined as $(1.10 \pm 0.26) \mu M$, which is in good agreement with the value determined for the radioactive label $(0.98 \pm 0.10) \mu M$ and with published data $(1 \mu M)$ (35).
Fluorescent probing of highly modified, structured small ribozymes with two terminal labels

Next, we investigated whether one could directly observe the perturbation of structural elements of RNA that is induced by the attachment of a bulky fluorescent label. This information would help in choosing the optimal position(s) for labelling, so that the native 3D structure of the RNA is kept intact, and the RNA remains fully functional. Small local alterations cannot be detected directly by observation of unexpected FRET efficiency values, as the global fold will remain largely unchanged. In contrast, in-line probing provides structural information on the single-nucleotide level and should therefore be able to resolve small perturbations in the fine structure of an RNA.

We tested this hypothesis by probing several DAse ribozyme constructs derivatized with a 3'-biotin and labelled with a Cy3/Cy5-FRET pair in different positions, designed to unravel the role of individual structural elements in single-molecule FRET studies (Figure 2a). The DAse represents a small, compact and highly structured artificial ribozyme that catalyzes a cycloaddition reaction (36). Its 3D structure involves three helices, a nested pseudoknot (dotted lines in Figure 2a) and further complex structural elements (7,37). Although the dye positions were selected based on high-resolution crystallographic information, one cannot predict which positions are more intrusive than others. In fact, recent activity measurements on FRET-labelled DAases yielded rather surprising results (25).

Most of the constructs used here are chemically modified on both termini and, thus, not easily accessible to standard ($^{32}$P) labelling and probing. In these cases, the fluorescent labels already present in the structure are the only possibility to obtain structural data from in-line probing experiments. All ribozyme constructs were subjected to in-line probing at three different magnesium ion concentrations: 0 mM where the ribozyme is inactive and the tertiary structure is not properly formed, 80 mM where the ribozyme is highly active and which is the standard assay concentration, and 20 mM as an intermediate value (7).

The in-line probing pattern of each of the constructs was analysed and compared to the one derived from the unmodified 5'-32P-labelled wt ribozyme reference...
(Figure 2c). If the oligonucleotide is labelled on both termini, as in construct A, two probing patterns are generated simultaneously during the probing experiment. The Cy3 dye, which is attached to the 5'-end, leads to a cleavage pattern identical to that of the 5'-32P label, resolving all cleavage products truncated from the 3'-end. Their 5'-truncated counterparts can be visualized on the same PAGE gel by monitoring the Cy5-label that is attached to U50 (representing an appended 3'-terminal label). This duplexing facilitates observation of the complete RNA to be probed on a single gel without compromising with the resolution (Figure 2b).

Analysis of the DAse cleavage patterns indicates a compact and highly ordered structure for both, wild-type and construct A. In agreement with earlier Pb2+-probing studies (7), the following features can be detected using the 5'-labels (Figure 2d): rather strong cleavage in the upper bulge region (nucleotides 20–26) and in the upper tetraloop (nucleotides 33–35), while helix 3 is unsusceptible to cleavage (nucleotides 27–32, 36–39). Some (weaker) cleavage is seen in the lower bulge region (nucleotides 40–45). The 3'-label additionally reports very low cleavage for helix 1 (nucleotides 5–18) except for the tetraloop (around U11). The cleavage bands at U6, G8 and C9 are likely related to a high-affinity magnesium ion binding site in close proximity (38). In agreement with earlier data, helix 2 is the weakest of the three helices and shows significant cleavage (nucleotides 19–22, 46–49). Two earlier observed cleavage hotspots are also detected (C26, U42, see loading control, lane NR) that correspond to pyrimidine–purine junctions.

In summary, neither the overall structure of the ribozyme, nor any relevant structural detail is perturbed by the attachment of the two labels on the two termini, as the cleavage pattern of construct A is identical to that of the wild-type ribozyme. In agreement with this observation, construct A was found to have full catalytic activity, compared to the wild-type (Supplementary Table S2) (25).

**In-line probing of constructs carrying one terminal and one internal label**

The situation that both dyes are attached to the two termini of the oligonucleotide is not the common case for FRET constructs. Internal labels have been—at least to our knowledge—completely neglected in chemical probing experiments, mainly for two reasons: (i) site-specific internal labelling is far more laborious than terminal labelling, and (ii) the analysis of cleavage patterns derived from internal labels is more demanding. In contrast to the easy-to-interpret ladder-like patterns derived from terminal labels, internal labels will produce two ladder-like patterns that overlay on the gel and cannot be assigned to specific cleavage products without additional information (Figure 3b). Often, two cleavage products of the exact same length will occur upon cleavage at different positions. For example, a cleavage at G2 results in a 47-mer, as a cleavage at G47 does. The two products cannot be distinguished directly as they both carry the dye and have similar electrophoretic mobilities. Still, the bands do not superimpose perfectly, as they have different sequences or (like in our case), carry different modifications. Now, the probing pattern derived from the terminal label (showing only one type of truncations) can be used as internal standard to distinguish the 3'-truncated from the 5'-truncated sequences in the pattern created by the internal label. This can be done since all cleavage products that carry both labels have to be present in both scans and have to superimpose perfectly (both, in position and intensity) (Figure 3b). Therefore the image of the terminal label can be subtracted from the one obtained with the internal dye after normalization. Then, only those bands remain visible that originate from truncation products.

**Figure 3.** In-line probing analysis with one terminal and one internal label. (a) In-line probing analysis of a Cy35'-Cy5U33-labelled DAse construct B. The right image is a subtraction of the Cy3 scan from the Cy5 scan after normalization. Selected cleavage bands are annotated on the left (for the Cy3 scan) and on the right (for the subtraction image) of the gel. The position of the internal label is indicated by the dashed lines. (b) Schematic representation of the probing experiment. The panel labelled with Cy5–Cy3 represents a subtraction image with all values <0 set to zero. The yellow box indicates which cleavage products superimpose in both scans (indicated as yellow bands). The dashed box shows the cleavage products visible in the subtraction image.
that do not carry the terminal label. The probing pattern can subsequently be analysed without bias.

To investigate this situation, we synthesized constructs B and C. While B has a 5′-terminal and an internal label, C carries a 3′-terminal and an internal label (Figure 2a). In Figure 3a, left panel, one can immediately assign the position of the internal dye in construct B by the large gap in the alkaline digest ladder. The probing patterns obtained for construct B show that much information can be derived from those parts of the gel that require image processing prior to analysis (Figure 3a). The cleavage status of all nucleotides between G1 and G14 can only be assigned after subtraction of the 3′-truncated species that carry both dyes, as outlined in Figure 3b.

The overall cleavage pattern is found to be very similar to that of the wild-type. However, B exhibits some slight differences. The most significant one is the disappearance of the cleavage band at U42 at high magnesium ion concentration, indicating a different conformation at this nucleotide, compared to the wild-type. As U42 is a key player within the hydrogen bonding network inside the catalytic pocket, it is not surprising that construct B shows reduced activity (60%, compared to wild-type), while constructs A and C are fully active (Supplementary Table S2) (25).

The absence of cleavage in the upper tetraloop (U32–G35), where the internal dye is attached, is expected to have less influence, as those nucleotides are far away from the active centre of the ribozyme.

The cleavage pattern of construct C is essentially identical with the one obtained for the wild-type ribozyme. In this case, the only additional information obtained from the second label is the cleavage band at C49 that results from the appended dangling U50 (Supplementary Figure S2).

In-line probing of constructs with two internal labels

In the third possible case, both fluorescent dyes are attached to internal positions (construct D, see Figure 2a). Here, both scans contain biased regions (Supplementary Figure S3). The products resulting from cleavage between both dyes can be resolved without bias as they carry only a single dye and will therefore appear only in one channel. They will either be resolved directly, or can be visualized by subtraction as described above (Figure 4a). For cleavage products that carry both labels, however, an unbiased annotation is not possible.

For construct D, nucleotides U6–A41 (or 73% of the sequence) can be assigned, while no exact annotation is possible for all bands resulting from cleavage at G1–C5 and U42–C49 as they have similar electrophoretic mobility, carry both labels, and can thus not be dissected unequivocally by subtraction (Supplementary Figure S2). Often, however, one of the two possible interpretations is much more likely than the other, if one considers general structural knowledge about the RNA under investigation, as well as the probing patterns of related constructs. For construct D, analysis of the two scans (including the subtraction images) confirms the overall fold of the DAse in the region from U6–A41 (Supplementary Figure S3). Comparison of the biased region with the reference images of construct A–C shows significant differences (Figure 4b). The strong cleavage band in construct D (annotated with U42) is not present in any other construct. This band could be either annotated to U42 (the nucleotide directly next to the dye label) or to C5 (helix 1). Observing a single strong cleavage band in an extended helical structure with high thermodynamic stability is extremely unlikely taking into further account that the other possible cleavage site is under direct influence of the label and located in the bulge region that is known to be susceptible to cleavage. In addition, Figure 4b shows that a cleavage band for U45, visible in all reference constructs is missing in construct D. As this nucleotide is, like U42, part of the lower bulge, we conclude that the local structure in this region is significantly changed. This may explain the complete inactivity of this particular FRET construct (Supplementary Table S2) (25).

In summary, these results demonstrate that the fluorescent labels in RNA FRET constructs can report structural perturbations induced by the dyes themselves when subjected to in-line probing, although some conclusions, especially in direct proximity of the dyes, can only be drawn indirectly. While in no case the overall structure was disturbed, two constructs showed slightly altered probing patterns ascribed to structural changes inside the catalytic

![Figure 4. In-line probing analysis with two internal labels. (a) Schematic representation of the experiment. The panels labelled with Cy5–Cy3 and Cy3–Cy5 are subtraction images with all values <0 set to zero. Yellow bands in the superimposed image indicate cleavage products present in both scans. The dashed box shows the part of the sequence that can be analysed unambiguously. The dashed line shows the border above which only cleavage products carrying both labels are present. (b) In-line probing analysis of four DAse ribozyme constructs (A–D). The images show a section of the unmodified Cy3 scan from the gels visible in Figure 2d, 3a and Supplementary Figure S3. Below each image, the relative catalytic activity, with respect to the wild-type ribozyme, is given.](image)
pocket (Figure 4b), and only those two constructs had a reduced catalytic activity.

In-line probing of photocaged, fluorescently labelled oligonucleotides

After demonstrating that fluorescent in-line probing could identify non-intrusive positions for labelling, we turned the question around and asked whether it could also help in verifying the efficient, intentional destruction of higher order structure. We designed several photocaged DAse constructs E–H (all derived from the Cy55-Cy3U33 DA construct B) for future single-molecule studies of ribozyme folding dynamics (Figure 2a). In addition to the modifications already present in the uncaged constructs, these caged ribozymes are derivatized with one or two o-nitrophenylethyl (NPE) groups in key positions that are predicted to be essential for the correct folding—and thus for the activity—of the ribozyme (Supplementary Figure S1). The NPE groups can be cleaved upon irradiation with light to yield the unprotected ribozyme. In contrast to the situation in the preceding paragraph, the goal is now to find constructs where certain elements of secondary or tertiary structure are significantly changed, compared to the wild-type.

Constructs E–G were designed to investigate how an essential element of secondary structure, namely helix 2, could be most efficiently perturbed, while in construct H the tertiary structure is disrupted. Differences in the probing patterns of E–G as compared to B allow to gain insights into structural perturbation(s) caused by individual caging groups and can be further correlated to changes in the catalytic activity of the ribozyme. It should be noted that the presence of a caging group influences the electrophoretic mobility of the respective fragments, and the position of the caging group(s) can therefore be derived directly from the probing patterns.

As can be seen in Figure 5a, the probing pattern of construct E shows a high degree of similarity to B. In the presence of magnesium ions, only the cleavage band at nucleotide U20 (the one that carries the photocaging group) is intensified. The caging group apparently does not prevent the formation of helix 2, and the catalytic pocket seems to be largely intact. This correlates well with the significant residual activity of 30%, compared to the B (Supplementary Table S2) (39).

In construct F and the double-caged construct G, extended regions of very uniform cleavage are visible (U20–A27 and A40–C49), indicating destruction of helix 2 and the ordered bulge region (Figure 5a). The only two parts of structure that are found to be protected from cleavage (and should therefore be still intact) are helices 1 and 3. The fact that both constructs show no catalytic activity in the caged state is in agreement with this finding (39).

In construct H, the photocaging group is located at the exocyclic amino group of nucleotide C25, a functional group that forms a pseudoknot-Watson–Crick hydrogen bond with G2 at the 5′-end (dashed line in Figure 2a), and an important tertiary H-bond with U42 in the lower bulge, thereby creating a base triple that is required for activity (37). Caging should interfere with both interactions. The overall probing pattern of this construct indicates that all three helices are intact, but increased cleavage is detected on both sides of the internal bulge, suggesting a less ordered structure in this region. In addition, we observe strong cleavage bands in the G1–G4 area, suggesting that the pseudoknot has opened (Figure 5b; see Supplementary Figure S4 for more details). This construct was found to be almost completely inactive (~2%, relative to B) (Supplementary Table S2). Activity could be fully restored upon UV-irradiation and, thus, removal of the photocaging group (28).
DISCUSSION

Fluorescence is the preferred detection signal for most of today's studies on RNA folding, activity, and structure in vitro as well as in vivo. Although the attachment of large fluorescent dyes is a major intervention in the structure of the RNA molecule, it is often taken for granted that the structural integrity of the molecules is maintained.

In this study, we investigated two very different types of RNAs, with different size, modification status and properties. In both cases, information was obtained on the single nucleotide level. The lysine riboswitch aptamer from B. subtilis was evaluated for its binding characteristics for l-lysine. The results confirm the previously reported properties. Three regions inside the molecule could be detected that showed attenuation in the same range as observed with radioactive labels. Furthermore, the apparent Kd-value for l-lysine was found to be essentially identical to earlier reported values. Therefore, ligand binding studies on large RNA aptamers can generally be conducted using fluorescent labels.

There is a high demand for analytical techniques that allow secondary and tertiary structure analysis of highly modified RNAs. For the small, structured DAsel ribozymes we could obtain information about perturbations of the native structure by the introduction of various modifications. We could detect very subtle effects of FRET dyes introduced at internal positions on the probing band pattern, while the overall fold remained essentially the same. These small changes correlate well with the catalytic activity of the ribozymes. We are not aware of any other analytical technique that allows direct analysis of the structural effects of dye labels down to the single-nucleotide level. Indirect RNA probing techniques like SHAPE (3,4) cannot be used for this purpose, as they rely on enzymatic primer elongation, which may be severely hampered by the presence of internal dye labels.

We could also experimentally validate the structural consequences of photocaging. Whether or not a caging group influences the function of an RNA can often be easily determined —e.g. by measuring the catalytic rate acceleration or binding constant. These measurements, however, do not tell which secondary or tertiary interactions have actually been disrupted by the caging group, and whether or not the design goals of the researcher have been met. Using in-line probing with fluorescent labels, we could unambiguously demonstrate the intentional disruption of the tertiary structure (pseudoknot, base triple, construct H) with intact secondary structure (all 3 helices), as well as disruption of the secondary structure (helix 2, F and G) with concomitant breakdown of the tertiary structure.

Besides using fluorophores, two other methodological novelties were introduced here into chemical probing, namely the use of two different labels in one molecule, and the utilization of internal labels. While this can—in principle—also be achieved with radioactive labels, such approaches are laborious and cumbersome, and have therefore never become popular. Whereas the existence of a second label inside the molecule to be probed will almost always provide additional structural information, there is—from a probing perspective—rarely a benefit in incorporating a label internally. However, internal labelling is the typical situation in FRET measurements, and therefore the technique described here is highly suitable for the structural analysis of FRET constructs. We could demonstrate that overlapping ladder patterns derived from internal labels can be deconvoluted by simple image subtraction.

In conclusion, fluorescent in-line probing is a promising technique for the structural investigation of RNAs of various sizes. While exhibiting the same resolution as the commonly used radioactivity-based probing, it can be much more easily applied to the highly modified RNAs typically used in biophysical studies.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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