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Time-Resolved Hydroxyl Radical Footprinting of RNA with X-Rays

Yumeng Hao,
Johns Hopkins University, Baltimore, Maryland

Ryan Hulscher,
Johns Hopkins University, Baltimore, Maryland

Mollie C. Rappé,
Johns Hopkins University, Baltimore, Maryland

Tadepalli Adilakshmi,
Johns Hopkins University, Baltimore, Maryland

Sarah A. Woodson,
Johns Hopkins University, Baltimore, Maryland

Jen Bohon, and
Center for Synchrotron Biosciences, Case Western Reserve, Cleveland, OH

Sayan Gupta
Center for Synchrotron Biosciences, Case Western Reserve, Cleveland, OH

Abstract

RNA footprinting by hydroxyl radical cleavage provides ‘snapshots’ of RNA tertiary structure or protein interactions that bury the RNA backbone. Generation of hydroxyl radicals with a high-flux synchrotron X-ray beam provides analysis on a short timescale (5 ms to 100 ms), which enables the structures of folding intermediates or other transient conformational states to be determined in biochemical solutions or in cells. This unit provides protocols for using synchrotron beamlines for hydroxyl radical footprinting.

INTRODUCTION

This unit describes the use of a synchrotron X-ray beam to carry out hydroxyl radical footprinting of RNA. With a high-flux beam optimized for footprinting applications, the cleavage reactions typically require 1 to 50 ms of exposure to the X-ray beam. Hence, this method is suitable for probing kinetic folding intermediates or other transient conformational states. Moreover, X-rays readily penetrate cells, allowing the RNA conformation to be probed in the cell by hydroxyl radicals generated *in situ*. The protocols given here were developed to probe the folding pathway of ribosomal RNAs (Adilakshmi et al., 2008; Clatterbuck Soper et al., 2013; Hulscher et al., 2016; Latham & Cech, 1989), but are applicable to a variety of non-coding RNAs or RNA-protein complexes. This protocol is predominantly based on capabilities of a new facility for X-ray footprinting is operated by the Center for Synchrotron Biosciences at beamline XFP (17-BM) of the National

Synchrotron Light Source-II (NSLS-II) at Brookhaven National Laboratory. Additional capacity for X-ray footprinting is available at the Advanced Light Source (ALS) at Lawrence Berkeley National Laboratory (Gupta et al., 2014).

Equilibrium experiments are conducted by exposing the RNA to the X-ray beam using a sample holder and electronic shutter (see Basic Protocol 1). Kinetic experiments are carried out using a commercial rapid mixing apparatus modified to withstand high-flux X-radiation (see Basic Protocol 3). RNA folding reactions are initiated by rapidly mixing the RNA with buffer containing $MgCl_2$. After a defined interval, the RNA is pushed through a flow cell in the X-ray beam for a time needed to achieve the desired dose. The cleaved RNAs are analyzed by primer extension and gel electrophoresis or capillary gel electrophoresis (see Unit XX) (Hulscher et al., 2016). Alternatively, libraries for high-throughput sequencing may be prepared from the cleaved RNA. This allows footprinting data to be analyzed across the entire transcriptome (see Unit XX) (Kielpinski & Vinther, 2014).

CAUTION: Prospective users must inform themselves of the requirements for user access, training, and safety compliance at individual synchrotron facilities. It can take several months to arrange access to the beamline, and advance planning is essential for a successful beamline experiment.

NOTE: Care must be taken to avoid introducing ribonucleases into the samples. This can usually be achieved by purchasing good-quality machine-packaged disposable plasticware, using gloves while handling samples and preparing solutions, and storing disposables in clean, dust-free containers. All solutions should be prepared with deionized water (18 MΩ) that is free of pyrogens and organics (e.g., tissue culture grade). Although less desirable, water treated with diethylpyrocarbonate (DEPC; *APPENDIX 2A*) may be used if a source of nuclease-free water is not available. Solutions should be sterilized by filtration (0.2-μm filter) or autoclaved.

NOTE: Samples within the experimental enclosure, or « hutch » should be shielded when the beam shutter is open, if levels of scattered radiation are detectable. Even small levels of exposure can undesirably elevate background RNA cleavage.

STRATEGIC PLANNING

These experiments require access to a synchrotron X-ray beamline that has been suitably equipped for footprinting studies. Requirements for the beamline configuration are discussed briefly at the end of this unit and elsewhere (Bohon et al., 2014). Protocols in this unit describe experiments conducted at beamline 17-BM (XFP) at the NSLS-II, using equipment available through the general user program. Information regarding facilities at XFP can be obtained at https://www.bnl.gov/ps/beamlines/beamline.php?r=17-BM and at http://csb.case.edu/xfp.html.

Synchrotron experiments must be planned 2 to 4 months in advance to allow users sufficient time to complete safety and equipment training before beginning work. It is essential to ensure that the required equipment is available and, if necessary, to allow for construction or

installation of additional equipment. Consultation with the beamline scientist is strongly encouraged.

Before undertaking experiments at the synchrotron, it is important to determine whether the RNA or RNA-protein complex of interest can be studied effectively by hydroxyl radical footprinting, and whether it is best to conduct the footprinting experiments in the cell or on purified complexes. Important considerations are whether the RNA or process of interest can be recapitulated in the test tube, and whether there exists an appropriate system for expressing the RNA of interest. For RNAs that can be refolded in the test tube, preliminary footprinting experiments may be conducted with Fe(II)-EDTA, which also generates hydroxyl radicals (*UNIT 6.5*). The cleavage patterns induced by Fe(II)-EDTA and X-rays are almost indistinguishable, although Fe(II)-EDTA reactions proceed more slowly. The experimental conditions (i.e., temperature, Mg^{2+} concentration, and protein concentration) should be adjusted to maximize the extent of protection. When combined with rapid mixing, in vitro X-ray hydroxyl radical footprinting can probe structural changes in 10 ms, which is one of its important advantages. Another advantage of X-ray footprinting is that RNA can be probed in a variety of cell types with little or no alteration to the culture conditions.

It is recommended that one begins with X-ray footprinting experiments under equilibrium conditions (see Basic Protocols 1 and 2). These experiments can be used to confirm that the optimum conditions have been selected, and establish the equilibrium parameters of the folding transition. The required X-ray exposure time for the sample must be determined from a dose-response curve and consideration of the current in the synchrotron ring at the time of the experiment (see Support Protocol 2). This information is indispensable in planning time-resolved experiments (see Basic Protocol 3). For in-cell X-ray footprinting, cells should be cultured and harvested under well-controlled conditions that are known to maximize the functional states of the RNA of interest.

BASIC PROTOCOL 1: EQUILIBRIUM X-RAY FOOTPRINTING OF RNA

This protocol is useful for performing titrations with Mg^{2+}, Na^{+}, urea, or protein. A temperature-controlled steel multi-sample holder is used to place up to 23 samples in the X-ray beam. An electronic Uniblitz® shutter (Vincent Associates) controls the exposure time for each sample (see Commentary, Figure 11.6.1).

Materials

Purified RNA (10 μM in 1 × CE; 10–100 pmol) prepared by *in vitro* transcription or frozen cell culture in 5–10 μL aliquots (see Support Protocol 1)

CE buffer, pH 7.5 (see recipe) or other suitable buffer (see Critical Parameters)

1 M $MgCl_2$ (*APPENDIX 2A*; optional)

Precipitation cocktail (see recipe)

100% ethanol

1.5-mL and 0.5-mL microcentrifuge tubes with captive screw caps and O-ring seals (Rainin)

Aluminum foil of varying thicknesses used to attenuate the beam

Alexa 488 stock solution in water (~200 μM)

0.2 mL Brandtech thinwall PCR tubes (Thermo-Fisher #13-882-58)

Small fluorimeter and 1 mL cuvettes for measuring Alexa 488 fluorescence

Temperature-controlled heating block or water bath

CAUTION: Cacodylic acid is an arsenic compound and is toxic.

XFP configuration for high-throughput sample exposure

The multi-sample exposure apparatus is made from a steel block with 23 slots shaped for mounting 0.2 mL PCR tubes along the horizontal axis of a motorized stage (see Commentary, Figure 11.6.1). A vertical motion stage is available for adjusting the apparatus to changes in beam position. Exposure time is controlled via a mechanical Uniblitz® (Vincent Associates) X-ray shutter, that is physically limited to a minimum reliable exposure time of 10 ms. To provide the beam needed to fully cover the ~2.5 mm diameter sample formed by 5 μL of solution held by surface tension in the bottom of these tubes, the apparatus is mounted at an endstation position downstream of the focal position. A diamond detector (see Commentary) is used to measure beam profile and flux prior to the experiment. A detector for automatic alignment is fixed in the beam path directly behind the apparatus; a Python routine enables scanning through the vertical and horizontal dimensions to automate centering of the slots in the beam. Note that the uniformity of the beam as possible and full illumination of the sample are critical parameters for obtaining reproducible data. For a 5 μL sample, the FWHM of the beam diameter should be ~2.5 mm. A series of Peltier coolers and a flow-through direct cooling/heating recirculating water bath system maintains the sample temperature from −30 C to 45 C. (Note that the water must be flowing prior to use of the Peltier coolers to avoid overheating.) A Python-based graphical user interface interacting with an underlying EPICS controls infrastructure allows programming of the apparatus for automated motion and shutter control once the desired parameters are entered.

At XFP, the beamline apparatus is configured by the beamline staff.

Verify alignment and calibrate beam by measuring Alexa dose-response

1. Insert an empty PCR tube into the first hole of the multi-sample holder, with the cap open and the open end facing the beam.
2. Engage the beamline interlock system, exit the hutch, and enable the X-ray beam.

   Experimental hutches at the NSLS-II are equipped with an interlock system to prevent accidental exposure of personnel. Procedures for enabling the beam are established by the synchrotron facility administration.
3. Confirm alignment of the beam by irradiating the empty tube for ~750 ms; re-enter the hutch and retrieve the tube to observe the position of the beam mark on the tube.

The burn mark on the tube should be centered at the bottom of the tube. Irradiation time is determined by the beam flux density absorbed by the tube. This may vary at different beamlines.

4 Dilute the Alexa 488 stock solution 1:100 into 0.5 mL 1x CE, culture medium, or other buffer to be used for the experiment. The final Alexa concentration should be adjusted as needed to achieve a fluorescence intensity approximately 100 times above the noise threshold when diluted 1:200 in measurement buffer.

5 Place 5 μL aliquots of the diluted Alexa 488 solution at the bottom of 15 0.2 mL PCR tubes.

6 Place each tube into the multi-sample holder, with the caps open and with the open end facing the beam.

7 Program the shutter controller to expose the samples to the beam for varying periods of time (*eg,* 0 to 50 ms). The first sample of each set should be left unexposed, as a control.

It may be necessary to place a thin sheet of aluminum in front of the sample to attenuate the beam in order to achieve the desired dose. This depends on radical scavengers in the sample or buffer (the less scavengers you have, the more attenuation you are likely to need)

8 Exit and interlock the hutch and open the shutters.

9 Run the Python program to expose the samples to the beam for the desired time periods, using the sample shutter and sample holder controller. Repeat a series of 5 exposure times three times for a total of 15 samples.

10 Disengage the shutters and enter the hutch to remove the samples.

11 Blank the fluorimeter with the same buffer used to the dilute the samples. Measure the fluorescence intensity of each sample by diluting 4 μL of the sample solution into 0.8 mL buffer.

12 Plot the logarithm of the relative fluorescence intensity, log (I_t/I_0), *versus* exposure time *t* to obtain a dose response curve (in triplicate).

13 If necessary, change attenuation levels and repeat steps 5–12 to obtain the desired dose range.

14 Once you are satisfied with the exposure conditions, bring the multi-sample holder to the temperature appropriate for the subsequent experiments.

If the apparatus is used with frozen samples, it is prudent to reconfirm the tube alignment in step 3 subsequent to reaching the working temperature.

Prepare RNA samples

15 Thaw the 10 μM RNA solution at room temperature and warm at 50 °C for 5 min if desired, to disrupt any aggregates. Microcentrifuge 15 sec at maximum

speed. Alternatively, prepare frozen aliquots of cell culture under the desired growth conditions (see Support Protocol 1).

Samples should be prepared and shipped to the beamline one or two days in advance. Store at −80°C when not in use.

16 Dilute 2 μL RNA solution (20 pmol) with 48 μL of the appropriate buffer (typically CE buffer), bringing the total volume to 50 μL. Vortex the samples and microcentrifuge 15 sec at maximum speed.

17 Label twenty 0.2-mL PCR tubes. Add 2.5 μL RNA (1 pmol) from step 17 to each tube.

18 Add the desired reagents (2.5 μL) to each tube.

For example, to measure the Mg^{2+}-dependence of RNA folding, prepare a series of solutions containing 2× $MgCl_2$ in CE buffer. Add 2.5 μL of 2× $MgCl_2$ to eighteen samples. Add 2.5 μL of CE (no Mg^{2+}) to two samples to serve as unfolded controls.

The total volume of each sample should not exceed 5 μL, to ensure that the entire sample remains within the cross-section of the beam.

19 Refold the RNA by incubating the samples at the desired temperature until the folding reaction has reached equilibrium, or by heating 1 to 2 min at 85° to 95°C and cooling to the desired temperature.

Prolonged incubation of RNA at temperatures >50°C should be avoided to minimize hydrolysis.

Expose RNA to X-ray beam

20 Set aside one of the samples without $MgCl_2$.

This sample will not be placed in the X-ray beam and will serve as an unexposed control.

21 Place the open tubes in the multi-sample holder with the large end facing the beam.

If whole cells are to be irradiated, they should be kept at −80 °C or on dry ice until placed in the sample holder, which should be chilled to −30 °C. If the cell pellets are allowed to soften, the RNA will deteriorate.

22 Interlock and exit the hutch, and expose the samples to the beam as in steps 8–10.

Each sample should be exposed for the same time. Exposure times are typically 10 ms for liquid samples (depending on attenuation) and 10–500 ms for frozen cells at XFP. The optimal time should be determined from a dose-response experiment (see Support Protocol 2).

23 Re-enter the hutch and remove the samples from the holder. To RNA solutions, add 15 µL distilled water, 5 µL precipitation cocktail, and 75 µL of 100% ethanol. Store the samples at −80 °C until analysis. If using frozen cell pellets, immediately place cell pellets on dry ice and store at −80 °C until they are ready to be analyzed.

BASIC PROTOCOL 2: X-RAY FOOTPRINTING OF LIVE BACTERIAL CULTURE

This protocol is for footprinting RNA in log-phase *E. coli* cells, but can be readily adapted for cells grown under other conditions or for biochemical solutions. A pump directs the culture through a narrow silica capillary placed in the X-ray beam and then to a fraction collector (Figure 11.6.2). The X-ray exposure time is determined by the flow rate.

Materials

E. coli cells (MRE600 or desired strain) streaked on LB agar and grown overnight.
RNA solution (10 µM in 1 × CE) prepared by *in vitro* transcription (optional)

1 L sterile LB broth or other suitable medium

0.5 L RNase-free water for rinsing flow path

0.2 L 100% ethanol for rinsing flow path

Alexa 488 stock solution in water (~200 µM)

15 mL culture tube with cap

2 or more 250-mL flasks with baffles and magnetic stir bar (one for each experiment plus one spare)

Shaking incubator

Visible spectrometer for measuring the optical density of the cell culture

Disposable cuvettes

Constant temperature water bath to hold 250 mL flask, or chemostat, plus magnetic stirrer

Vici M50 positive displacement pump or syringe pump capable of 5–10 mL/min flow rate

Flow cell with silica capillary (0.53 mm internal diameter), connected to pump and to fraction collector

Fraction collector with racks to hold 1.5 mL tubes

Aluminum foil of varying thicknesses, used to attenuate the beam

1.5-mL microcentrifuge tubes

Microcentrifuge (preferably refrigerated).

XFP flow cell for exposing cell culture

The XFP *in vivo* flow cell apparatus consists of four main components: a water bath and stirring system used to maintain the culture at the desired conditions, a positive displacement pump (VICI®) to withdraw the sample from the culture and move it through the capillary and tubing system, a custom water-cooled flow cell, and a programmable fraction collector (Teledyne) that can handle up to 120 1.5 mL tubes. In addition, an upstream shutter enables protection of the sample and apparatus when exposure is not required. For X-ray exposure, a silica capillary is held in the custom water-cooled (4 °C) exposure cell mounted to an x–y stage for alignment; the flow cell is mounted such that the direction of flow is vertical. Exposure time for the sample is determined by the speed at which the sample is moved through the beam and the size of the beam.

It is critical that the X-ray beam covers the entire ID of the capillary so that the dose to the sample will be uniform. For this type of experiment, a combination of mirror adjustments and positioning of the sample exposure cell to a slightly unfocused position within the hutch at XFP enables use of a beam that is ~0.75 mm wide and 0.12 mm tall to fully cover the 0.53 mm ID capillaries that are preferred when pumping cell culture. A Python-based command line interface (interacting with an underlying EPICS controls infrastructure) allows programming of the apparatus for automated pump, fraction collector and shutter control.

Verify alignment and calibrate beam by measuring Alexa dose-response

1. Enable the X-ray beam as described in Basic Protocol 1.
2. To ensure that the capillary is aligned to the beam, align the flow cell without a capillary by moving the flow cell horizontally and observing the beam on the imaging detector downstream.

   The image should show that the beam is large enough that a uniform signal is observable when centered and that movement of 50 microns in any direction will not move the capillary out of the peak intensity region of the beam.
3. Insert the capillary into the flow cell by sliding it down between the steel slits and attach capillary to flexible tubing leading to pump (top of capillary) and fraction collector (bottom of capillary).

   The capillary should be snug in the flow cell to avoid misalignment due to placement; if it slides too easily, the steel slits must be adjusted.
4. Flush the system with water and appropriate media (at least 3 mL)
5. Measure the dose-response of Alexa 488 to gauge the beam intensity if desired. Dilute the Alexa stock 1:1000 in minimal media, and deliver 1.2 mL, collected in 400 μL fractions. Combine 300 μL of the central fraction with 600 μL media and measure the fluorescence as in Basic Protocol 1.

Prepare bacterial culture

6 One night before the experiment, place 2 mL sterile LB broth in a 15-mL culture tube and inoculate from a single MRE600 colony. Grow overnight at 37 °C with shaking.

7 On the day of the experiment, place 75 mL sterile LB broth in each 250-mL culture flask.

8 Inoculate each flask 1:500 to 1:1000 with the overnight culture.

Stagger the inoculation of flasks so they will be ready for use at different times throughout the day.

9 Place flasks in the shaking incubator and grow at 37 °C until it reaches mid-log phase (OD_{600} = 0.6).

Measure the optical density of the culture using the disposable cuvettes and spectrometer.

10 When the culture has reached the desired growth stage, record the optical density of the culture and remove 2 0.75-mL aliquots in 1.5 mL tubes, to serve as « no hutch » controls.

These samples can be analyzed to check RNA quality before exposure to the flow path or to stray X-rays in the experimental hutch.

Expose bacterial cells to X-ray beam

11 Place the culture flask in the water bath in the experimental hutch and turn on the magnetic stirrer to aerate the culture.

12 Insert the tubing from the pump into the culture so that it reaches to the bottom of the flask. Make sure that the outlet is connected to the fraction collector, and fill the rack with 7–10 1.5 mL microcentrifuge tubes.

13 Using a programmable controller outside the hutch, turn on the pump and fraction collector, and deliver 5 mL culture. The effluent should be collected in 0.75 mL fractions.

A “no dose” or mock-treated control is obtained by passing the culture through the tube without turning on the X-ray beam.

The first two fractions will contain aged solution and should be discarded. Tubes 3–5 should contain fresh culture, depending on the internal volume of the flow path.

14 Interlock and exit the hutch, and enable the X-ray beam by opening upstream shutters. Expose the samples to the beam as in step 13.

The flow rate should be adjusted to achieve the desired exposure, typically between 0.5 and 5 mL/min (see Critical parameters). Aluminum foil may be placed between the sample and the beam to attenuate the exposure if needed.

Several exposure parameters should be tested to ensure optimum cleavage of cellular RNA.

15 Repeat step 14 with different flow rates or at different growth intervals until the desired number of samples have been collected.

Make sure that the fraction collector contains enough tubes to collect all of the culture, or reset the fraction collector for each condition.

16 Repeat step 13 without opening the shutter (optional).

These samples will serve as post-experiment unexposed controls, to diagnose any loss of RNA quality over the course of the experiment.

17 At the end of each set of exposures, disable the shutter, re-enter the hutch, and remove the irradiated samples from the fraction collector rack. Immediately harvest cells by spinning in a microcentrifuge 2 min at 14,000 x *g*. Decant the media, and freeze cells pellets in liquid nitrogen. Store the samples at -80 °C until analysis.

Cells should be harvested and frozen promptly (≤10 min) after X-ray exposure.

Clean up

18 Thoroughly rinse the pump, capillary, and tubing with distilled water to remove any traces of bacterial culture, followed by 100% ethanol.

19 Turn off and disconnect the water bath, magnetic stirrer, fraction collector and the shutter controller.

20 At the end of the experiments, complete all safety checks and disable the beamline.

BASIC PROTOCOL 3: TIME-RESOLVED X-RAY FOOTPRINTING OF RNA

This protocol describes a standard method for time-resolved X-ray-dependent hydroxyl radical cleavage of RNA. A KinTek(R) rapid mixing device is used to initiate the reaction of interest (such as Mg^{2+}-induced folding). After a programmed delay, the RNA is pushed into a flow cell and exposed to the X-ray beam. A progress curve is produced by varying the delay time. Samples are processed and analyzed as described in Basic Protocol 3.

Materials

CE buffer, pH 7.5 (see recipe) or other appropriate buffer (see Critical Parameters)

5–10 μM RNA stock solution

100% ethanol

Precipitation cocktail (see recipe)

CE20 buffer (see recipe)

Additional buffers or salts as desired (see Critical Parameters)

Rapid-quench apparatus, modified for X-ray footprinting experiments (e.g., RQF-3, KinTek®; see Commentary, Figure 11.6.3, and Support Protocol 3)

1-mL and 5-mL Luer-lok™ disposable syringes

Temperature-controlled heating block or water bath

1.5-mL microcentrifuge tubes with captive screw caps and O-ring seals (Rainin)

13-G needles

15-mL sterile disposable culture tubes

Refrigerated recirculating water bath

NOTE: It is important to become familiar with the operation of the rapid-quench apparatus (Figure 11.6.3) and the valve settings (LOAD syringes, LOAD sample, FIRE, and FLUSH). For RNA folding experiments, drive syringe B is loaded with buffer (e.g., CE or CE20 buffer), and the RNA sample is placed in the bottom right sample loop. Drive syringe A and the bottom left sample loop are loaded with CE or CE20 buffer. The third Quench syringe (C) is not used in the standard footprinting protocol. The plunger for the C syringe should remain fully depressed.

Adjust drive platform

1 Set up the rapid-quench apparatus as described in Support Protocol 3. Open a software routine that allows one to adjust the position of the drive platform (option 2 in the control software).

2 Use the remote actuate (small red) button on the stepper-motor to raise the platform.

The platform should just contact the plungers of drive syringes A and B when they are completely filled. One may calibrate and mark the desired starting position for the drive platform in advance.

Load drive syringes

3 If using a KinTek® apparatus, turn the top row of valves to the LOAD syringe position, and inject 1× CE buffer using a 5-mL disposable syringe. Work the solution back and forth to remove bubbles. Fill the A and B drive syringes in the same manner.

4 Turn the top valves so they are aligned vertically (FIRE). Remove the 5-mL disposable syringes.

Anneal RNA

5 Add 112 μL 1× CE buffer to 28 μL RNA stock (140 μL final volume; 2 μM RNA). Mix well (vortex) and microcentrifuge briefly at maximum speed.

6 If desired, anneal the RNA by incubating 10–20 min at 50 °C or 1 min at 95°C in a heating block. Microcentrifuge 15 sec at maximum speed.

This yields enough sample for a “priming shot” and nine experimental trials.

7 Prepare a prefolded RNA sample by mixing 10 μL RNA stock with 15 μL water and 25 μL 2× folding buffer (*eg,* CE20).

Load sample syringes

8 Using a 1-mL disposable syringe, pull the plunger back and draw in 0.3 mL air. Then, draw up the entire diluted RNA sample prepared in step 6.

The final drop of RNA solution can be transferred to the syringe tip using a pipet. The air in the syringe will be used to push the entire sample into the sample loop of the rapid-quench apparatus.

9 Turn the two bottom sample loop valves to the LOAD sample position. Attach the RNA sample syringe to the right sample port, and fill the tubing between the port and the valve. Leave the 1-mL syringe in place, as it will be used for subsequent experiments.

The top valves should be in the FIRE position. Be sure that the vacuum line is disconnected from the exit tube.

10 Using a 1-mL syringe, fill the left sample loop with CE buffer.

11 Turn the bottom valves to the FIRE position.

All valves should be in the FIRE position, except for drive syringe valve C, which remains in the LOAD position.

12 Remove the cap from a spare 1.5-mL screw-cap microcentrifuge tube and punch a hole through the cap using a 13-G needle. Thread the exit line of the quench-flow through the cap (it should fit snugly). Screw an empty microcentrifuge tube onto the exit line adapter, and place it in a tube rack on the aluminum shelf under the rapid-quench apparatus.

This tube will be used to collect waste.

An initial shot primes the drive lines and should be performed every time the drive syringes or sample loops are filled.

CAUTION: *The sample syringe containing RNA should be covered with either leaded Plexiglas or separate Plexiglas and lead shields to protect the sample from X-rays.*

Load sample loops and fire a priming shot

13 Turn the upper and lower valves to LOAD sample. Advance exactly 10 μL from each sample syringe into the left and right sample loops, respectively.

The valve and sample loop should be precalibrated and marked to indicate the proper position of the fluid meniscus. The amount of solution is determined by the volume of the sample loops (see Commentary).

14 Turn all valves to the FIRE position.

The valve to drive syringe C should remain in the LOAD syringes position, unless a quench solution is used.

15 Open the software routine for the time-resolved footprinting experiment. Set speed and distance of the stepper motor to achieve the desired folding delay and exposure times. For the dummy shot, enter a delay time of 0.01 sec.

Each push of the drive platform is defined by the speed (rpm) of the stepper motor and a distance (the number of turns of the motor).

16 Once the parameters are set, type “G” at the controller keyboard to fire the drive motor.

Before firing, be sure that a microcentrifuge tube is attached to the exit line.

Flush mixing and exit loops

17 Turn the lower valves to FLUSH, and allow any sample remaining in the exit line to drain into the waste or sample collection tube.

Alternatively, turn the valve under syringe C 180 degrees, and expel the sample from the exit line by blowing air through port C with an empty 1-mL syringe. Close the valve (return to LOAD syringes).

The lines of the rapid mixing apparatus are flushed and rinsed after each shot.

18 Gently unscrew the microcentrifuge tube from the exit line.

19 Attach the vacuum to the exit line using the Luer adapter.

If the valves are not set to the FLUSH position before attaching the vacuum, the contents of the sample syringes will be aspirated into the waste.

20 With values in the FLUSH position, dip the wash inlet (tubing with a T joint protruding from the bottom of the rapid-quench apparatus) into a 15-mL tube of deionized water for 5 sec, and then into a 15-mL tube of 100% ethanol for 5 sec. Allow the vacuum to pull air through the system for 30 sec to dry.

The vacuum will draw the solutions through the mixing and exit loops.

21 While holding the exit line, carefully detach the vacuum. Hang the end of the vacuum line (with Luer adapter) in an empty, disposable 15-mL tube to avoid contaminating the work area. Screw the waste tube onto the exit line.

CAUTION: *The exit line and the end of the vacuum line should be handled with care to keep them free of ribonuclease.*

In a typical experiment (20 reactions), an unexposed control and a reaction without Mg^{2+} are acquired first (reactions 1 and 2). The left side drive syringe and the bottom left sample loop are then refilled with CE20 or the desired 2× folding buffer, and 16 folding time points (reactions 3 to 18) are acquired by varying the folding delay time. Finally, two controls with prefolded RNA are performed (reactions 19 and 20).

22 Dispense 20 μL precipitation cocktail into each of 20 screw-cap microcentrifuge tubes. Label tubes 1 through 20 (or as desired). Recap tubes and hold at room temperature until needed.

These tubes will be used to collect samples. It is important to avoid introducing nucleases or other contaminants.

Perform a “no exposure” control

23 Screw tube no. 1 onto the exit line adapter. Set the valves to LOAD sample, and inject 10 μL RNA and 10 μL CE buffer into the right and left sample loops as in steps 13, and 14.

24 Set the folding delay time to 0.01 sec, and repeat steps 15 and 16.

25 Expel any sample remaining in the exit line into the collection tube as in step 17.

26 Remove the tube from the exit line, and flush lines with deionized water and 100% ethanol as described in steps 18 through 21.

27 Add 600 μL of 100 % ethanol to the expelled sample, recap, and store at 4° to −20°C.

It is important that the screw caps form a leak-proof seal to prevent loss of sample during shipment.

Perform a control with unfolded RNA

28 Attach tube no. 2 to the exit line. Reload the sample loops as in steps 13 and 14, except this time the interlock system should be enabled as the user exits the hutch.

29 Enter a delay of 0.01 sec in the controller software.

30 Turn on the X-ray beam when the interlock safety alarm has turned off.

31 Once the beam is on, fire the rapid-quench apparatus (type “G”). Turn the beam off as soon as the software displays a message indicating that the experiment has finished.

The time that the beam is on should be kept to a minimum to limit X-ray damage to samples and equipment.

32 Collect the sample and flush the mixing apparatus as in steps 17 to 21. Precipitate the RNA as in step 27.

Exchange buffer in drive syringe A

33 Close the top valves to drive syringes A, B, and C (LOAD syringes). Close the bottom left valve (180° from LOAD sample) and open bottom right valve (as in LOAD sample). Carefully remove the syringe containing RNA, and set aside for future use.

> To probe Mg^{2+}-induced folding, the CE buffer in the left side of the mixing apparatus (drive syringe A) must be exchanged for CE20 buffer.

34 Attach the vacuum to the exit port and open the bottom left valve (LOAD sample). To empty drive syringes, turn the bottom and lower valves to FIRE. Slowly depress the plunger of syringe A and B until the syringe is empty. Open the lines connecting drive syringes to air (opposite of FIRE).

35 Disconnect the vacuum, and return all valves to LOAD syringes.

36 Using the control software, move the drive platform up as in steps 1 and 2.

37 Fill drive syringe A with CE20 buffer as described in steps 3 and 4. Fill drive syringe B with additional CE buffer.

38 Load the bottom left sample loop with CE20 buffer (using a fresh 1-mL syringe) as in steps 9 through 11. Replace the syringe containing RNA on the right sample port.

39 Perform a priming shot and flush the mixing and exit lines, as described in steps 13 to 21.

Expose samples with variable folding delays

40 Using collection tubes no. 3 to 18, acquire time-resolved data by repeating steps 28 to 32 for each sample. Vary the length of the folding delay by entering the appropriate value into the software before firing each shot.

> If the folding delay is longer than the time required for the interlock safety shutter to open (3 sec), begin the folding routine, then turn on the X-ray beam when 3 sec of the delay remains. A standard laboratory timer can be used to measure folding delays of ≥10 sec.

41 When the first aliquot of RNA is expended (typically after the ninth sample), prepare the second aliquot as in steps 5 to 7. Remove the right sample syringe, and replace it with a fresh 1-mL syringe of RNA (steps 8 to 9). Fire a priming shot, then repeat step 40 for the remaining reactions, no.10 through 18.

42 When the experimental reactions are complete, open the right sample syringe to the vacuum (it should point to the right), and aspirate any remaining RNA. Flush the lines as in steps 17 to 21. Remove the vacuum from the exit line, and the 1-mL syringe from the right sample port.

Alternatively, the sample syringe can be removed and remaining RNA recovered before the system is flushed with water and 100% ethanol.

Acquire controls with prefolded RNA

43 A control sample should be prepared under conditions in which the RNA is known to fold completely (see step 7).

44 Load the prefolded RNA (50-μL) into the right sample loop, and fire a priming shot (steps 8 to 9, and steps 13 to 21).

45 Using collection tubes no. 19 and 20, perform two shots with a 0.01-sec folding delay, as in steps 28 to 32.

46 Store samples at −20°C or on dry ice.

Samples should be stored at −20° or −80°C until they are ready to be analyzed by primer extension (see Basic Protocol 3). This is typically done in the investigator's home laboratory.

47 When the experiment is complete, thoroughly flush and disassemble the rapid-quench apparatus (see Support Protocol 3). At the end of the day, complete all safety checks, and disable the beamline.

SUPPORT PROTOCOL 1: PREPARATION OF FROZEN BACTERIAL CULTURE

Bacteria may be cultured in the desired conditions at the home institution, and frozen in small aliquots prior to exposure to the X-ray beam. This protocol is for preparing *E. coli* cells for X-ray footprinting, but may be adapted for yeast or mammalian cells.

Materials

E. coli (MRE600 or other strain), streaked on an LB agar plate or selective medium

3 mL sterile LB media in capped culture tube

50 mL sterile LB media in 250 mL flask with baffles

TM buffer (10 mM Tris-HCl, pH 7.5, 1 mM $MgCl_2$), at 4 °C

Shaking incubator

Visible spectrometer with cuvettes

Ice/ethanol bath

Preparative centrifuge with rotor for 50-mL tubes (*e.g.*, Beckman JS13.2 or JA-20)

Microcentrifuge with rotor for 0.2 mL tubes

0.2 mL thinwall PCR tubes (BrandTech #781305)

Dry ice/acetone bath or liquid nitrogen in small dewar

NOTE: Biohazardous or infectious materials must be labeled and shipped in compliance with governmental regulations. Consult with the biological safety officer of your home institution and the receiving institution before planning to ship cultured cells.

Grow bacteria to early mid-log phase

1 Inoculate a 5 mL culture from a single colony and grow overnight at 37 °C with shaking.

Add selective antibiotics to the medium if required.

2 Use the overnight culture to inoculate 50 mL culture medium with selective antibiotics (1:100 to 1:250) in a 250 mL flask. Grow with shaking at 37 °C.

3 Grow the culture to OD_{600} = 0.4 to 0.6, measuring the optical density of the culture every 30 minutes using the spectrometer.

4 Immediately chill the culture by swirling the flask in an ice/ethanol bath for 30 s, taking care not to freeze the culture.

Dispense cells for X-ray footprinting

5 Transfer the cells to pre-chilled 50 mL centrifuge tubes and centrifuge at 5,000 rpm for 10 min at 4 °C.

6 Decant supernatant and gently resuspend cells in 20 mL chilled TM buffer.

7 Harvest cells as in step 5 and decant supernatant.

This wash step reduces levels of extracellular ribonucleases.

8 Gently resuspend cells in 300 μL TM buffer (~6 μl/μg wet cells or $1.5 \cdot 10^{11}$ cells/ml).

9 Dispense 5 – 10 μL aliquots of cell slurry into 0.2 mL tubes, keeping cells on ice.

Briefly spin tubes if needed to ensure that each sample is at the very bottom of the tube. Samples on the sides of the tubes will not be in the path of the beam.

Use 1 – 20 cell pellets for each analysis depending on the quantity of RNA required.

10 Snap freeze cell pellets in a dry ice/acetone bath or liquid nitrogen.

11 Store samples at −80°C until shipment to the beamline.

12 Package samples in ziplock bags for shipment on dry ice, ensuring that samples are entirely surrounded with dry ice. Store at -80 upon arrival until use.

SUPPORT PROTOCOL 2: DETERMINE OPTIMAL EXPOSURE TIME

The optimal time that samples should be exposed to the X-ray beam is determined by measuring the fraction of RNA that is cleaved after variable exposure times, for a particular

beam current and attenuation. The dose-response should be measured in the buffer or medium used for RNA folding experiments to control for the effect of solutes.

Additional Materials (also see Basic Protocols 1 and 2)

5–20 μL 1 pmol/μL 5′32P-labeled sequencing primer complementary to the 3′ end of the RNA of interest (*UNIT 6.1*)

Irradiated RNA (0.5 – 1 pmol; see Basic Protocol 1)

Mock exposed RNA (0.5 – 1 pmol; see Basic Protocol 1)

Untreated RNA control (250 nM) (see Support Protocol 2)

Exposed and mock exposed frozen cell pellets (optional; see Basic Protocol 2)

RNeasy Mini RNA isolation kit (Qiagen; if using RNA from cells)

5× First Strand buffer (Invitrogen; see recipe)

Superscript III reverse transcriptase (Invitrogen Life Technologies)

55° and 65°C water baths or heating blocks

Ethanol and salt for RNA precipitation

2× formamide loading buffer (see recipe)

Additional reagents and equipment for preparing and running 6% or 8% polyacrylamide sequencing gel (*APPENDIX 3B*)

NOTE: The following steps are carried out on ice except where indicated. Thawed reagents should be kept on ice throughout.

Isolate total RNA from cell pellets if using

1 If performing X-ray footprinting in cells, isolate total RNA from each sample using an RNeasy Mini kit (QIAGEN), following the manufacturer's instructions. For each preparation, combine two or three identical 5 μL pellets from Basic Protocol 1 or 3 0.75-mL fractions from Basic Protocol 2 for each prep, to increase the yield of RNA.

Trizol may also be used to isolate total RNA.

Anneal complementary primer to RNA

2 If desired, prepare the RNA template by denaturing at 95 °C for 1 min and immediately placing it on ice.

This step is only necessary for highly structured RNA templates.

3 For each sample, mix primer and template in a 0.5-mL microcentrifuge tube (total volume 12 μL):

10 μL RNA (0.1 μM *in vitro* transcript or 0.5 to 2 μg total cellular RNA)

1 μL 1 pmol/μL 5′ ^{32}P-labeled sequencing primer

1 μL deionized water.

4 Incubate in a water bath or heating block at 65 °C for 3 min and place immediately on ice. Microcentrifuge briefly at maximum speed before opening tubes. Keep samples on ice.

Some primer/template combinations require heating 2 min at 80 °C.

Extend primer with reverse transcriptase

5 Prepare a RT cocktail by mixing the following (volumes are per sample):

4 μL 5 × First strand synthesis buffer

1 μL 100 mM DTT

1 μL 10 mM dNTP mix

1.5 μL water

0.5 μL Superscript III.

Add the enzyme last. Resuspend enzyme thoroughly in the buffer, but do not vortex and avoid foaming.

6 Begin primer extension by adding 8 μL RT cocktail to each 12 μL reaction (steps 3 and 7). Immediately place each tube in a water bath or heat block at 55 °C for 15 min or up to 1 hr for long RNAs.

7 Stop reactions by adding 20 μL 2× formamide loading buffer or by heating 85 °C for 5 min.

8 Load the cDNA samples on a large 6% or 8% polyacrylamide sequencing gel, and run the gel electrophoresis for 2 h at 55 W or until the primer has reached the bottom of the gel.

The samples should correspond to a series that includes an unirradiated control and 3–5 samples exposed to the X-ray beam for different lengths of time.

It is not necessary to prepare sequencing ladders to measure the extent of RNA fragmentation, but sequencing ladders may be included if molecular weight markers are desired.

9 Disassemble the gel and dry under vacuum. Expose the gel to a Phosphorimager screen long enough so that the full-length RNA appears as a dark band, but does not saturate the Phosphorimager screen.

10 Use the volume integration feature of the image analysis software to quantify the amount of uncut RNA remaining after cleavage. Plot the fraction of full-length (*FL*) cDNA remaining at each exposure time versus time *t* and fit to an exponential decay, $[FL(t)/FL(0)] = \exp(-kt)$.

The decay rate k is used to calculate the exposure time t needed to achieve the extent of RNA cleavage appropriate for the sequencing method to be used (see Critical Parameters).

The dose depends on the flux density of the beam as well as exposure time. The exposure time must be adjusted to account for variations in the beam current.

SUPPORT PROTOCOL 3: SET UP RAPID-QUENCH MIXING APPARATUS

A modified KinTek® RQF-3 rapid-quench apparatus can be used for X-ray footprinting (see Commentary). The mixing apparatus must be installed at the beginning of the experiment. Users should consult the manufacturer's literature for more detailed information on the maintenance and use of the rapid-quench device. This protocol should be adapted to suit equipment available at individual facilities.

Additional Material (also see Basic Protocol 3)

Detergent (e.g., Absolve, NEN)

Plastic-backed absorbent bench paper

Diaphragm vacuum pump (details)

Vacuum/vent filter, 0.2 μm (Millipore Millex 50 mm, or equivalent)

Side-arm flask with one-hole stopper and Teflon tube

Thick-walled soft tubing (e.g., Tygon, Nalgene) to fit Teflon tube

Adapter (male M6 to male Luer) to connect soft vacuum tubing with $^{1}/_{16}$ -in.-o.d. polypropylene tubing (exit line of rapid-quench)

5-mL syringes

Install rapid-quench apparatus

1 Mount the KinTek® rapid-quench apparatus (in polycarbonate water jacket) on the stepper motor frame (Figure 11.6.3).

2 Use the provided bolts to attach a horizontal aluminum plate on the Unistrut frame, near the bottom of the mixing apparatus. Mount a second vertical aluminum plate on the rear of the rapid-quench box so that it rests against the first plate.

The horizontal plate will support sample tube racks. The vertical plate shields the sample exit lines from the X-ray beam.

3 Cover all exposed surfaces of the work area with plastic-backed absorbent bench paper to prevent contamination of hard surfaces in case sample is spilled.

4 Outside the hutch, turn on the controller and begin running the control software. If using a personal computer to interface with the controller, open the KinTek® software (or equivalent) in DOS and download the desired control program.

Calibration settings and mixing parameters for individual mixers can be stored between experiments and imported into the KinTek® control software.

Connect water bath and vacuum lines

5 *Optional:* Remove nucleases by soaking sample ports and internal tubing for 1 hr or overnight in detergent. Before use, remove all traces of detergent by thorough rinsing with RNase-free water followed by 100% ethanol.

6 Inside the hutch, connect the recirculating water bath to the jacket surrounding the rapid-quench apparatus. Set the temperature as desired.

7 Connect the vacuum pump to the side arm of a 0.5-L side-arm flask, through a 0.2-μm filter.

CAUTION: *This flask will collect the waste between kinetic shots. If the waste is hazardous, be extremely careful when handling liquid waste containers, and be sure that secondary trap and vacuum lines are secured.*

8 Attach thick-walled soft tubing to a Teflon tube in the top of the side-arm flask. Insert a male M6 to male Luer adapter into the opposite end of the tubing.

The vacuum tubing should be stored in a clean, covered box to minimize risk of RNase contamination. The adapter should fit snugly over the $^{1}/_{16}$ *-in.-o.d. tubing that is used as the exit line of the rapid-quench apparatus.*

9 Turn on the vacuum pump. Attach the vacuum to the exit line, and aspirate any solution from the sample loops and drive syringes.

Align mixing apparatus with X-ray beam

10 Secure the automatic alignment device to the registration pins on the back of the mixing box, and attach the output cable.

11 Enable the X-ray beam and align the sample capillary and exposure port with the beam.

If the beam is not aligned correctly with the exposure port, the extent of RNA cleavage may be drastically reduced. Although not necessary, it is prudent to check the alignment of the mixer at the end of an experimental session. This test will help troubleshoot the experiment in the event of poor results.

12 Remove the alignment device from the rapid-quench apparatus and extend the flight tube to the back of the rapid quench.

Failure to do so will prevent the samples from being exposed to X-rays during the experiment.

13 *Optional:* Manually check the alignment by taping paper over the exposure cell port on the rear of the rapid-quench apparatus. Expose the paper to the X-ray beam for 1 sec, and verify that the burn mark on the paper is centered in a depression caused by the outline of the exposure port.

The apparatus is now ready for use as described in Basic Protocol 3.

Clean up

14 Attach the vacuum to the exit line, and aspirate any remaining solutions from the sample loops and drive syringes. Leave the vacuum on.

15 With a fresh 5-mL syringe of deionized water, rinse out all the syringes and sample loops. Use the vacuum to aspirate each line after rinse.

16 Repeat step 15 with a 5-mL syringe of 100% ethanol.

17 Remove the vacuum line and turn off the pump and water bath. Remove the mixing apparatus from the support stand and store in a covered plastic box.

18 Exit the control software and shut off the controller.

SUPPORT PROTOCOL 4: CALIBRATE RAPID-QUENCH MIXING APPARATUS

Each mixing apparatus should be calibrated when first installed, or when tubing is changed. The parameters for the drive platform distances can be stored in the controller software. The performance of the rapid-quench mixing apparatus is evaluated visually using a water-soluble dye such as bromphenol blue.

Additional Materials (also see Basic Protocol 3 and Support Protocol 3)

0.25% (w/v) bromphenol blue in water

Dental mirror

Small flashlight

1. Set up a rapid mixing apparatus (see Support Protocol 3). Load drive syringes with water or CE buffer, as described (see Basic Protocol 3, steps 1 to 4).
2. Load 0.25% bromphenol blue solution into the right sample loop (instead of RNA), as described in Basic Protocol 3, steps 8 to 11. Fill the left sample loop with water or CE buffer.
3. Set up a mock footprinting experiment with a long folding delay (>10 sec) and a very long exposure time (10 sec). Install a microcentrifuge tube at the exit tube to collect the ejected sample.
4. Exit the hutch without enabling the beam. Type “G” to go.
5. Quickly re-enter the hutch. Inspect the flow of colored solution in the tubing using a dental mirror and flashlight. If the dye and water are not mixing correctly, empirically adjust the distance values in the KinTek® control program.

After the first push, the dye should advance up to the exposure cell, but should not enter it. The dye should mix uniformly with water. Once the "exposure" push starts, the dye should smoothly pass through the exposure cell and into the microcentrifuge tube.

REAGENTS AND SOLUTIONS

Use RNase-free deionized, distilled water in all recipes and protocol steps. For common stock solutions, see ***APPENDIX 2A****; for suppliers, see* **SUPPLIERS APPENDIX**.

CE (sodium cacodylate/EDTA) buffer, 1× (pH 7.5)

1.0 mL 1 M sodium cacodylate

20 µL 0.5 M EDTA, pH 8.3 (*APPENDIX 2A*)

Deionized water to a final volume of 100 mL

Filter sterilize through a 0.2-µm filter

Store up to 6 months at room temperature

CE20 buffer, 1×

Prepare as for 1× CE buffer (see recipe), but include 2 mL of 1 M $MgCl_2$ (*APPENDIX 2A*; final 20 mM).

Alexa 488 stock solution

Dissolve Alexa Fluor® 488 NHS (Succinimidyl Ester) (Invitrogen) in water to ~200 µm final concentration. Store solution at −20 °C wrapped in foil, and avoid exposure to light to minimize photobleaching.

Precipitation cocktail, 10×

8 mL 4 M NaCl (*APPENDIX 2A*)

0.5 mL 0.5 M EDTA, pH 8.3 (*APPENDIX 2A*)

100 µL 10 mg/mL carrier tRNA

150 µL 20 mg/mL glycogen

Bring to 10 mL with deionized water

Store in 1-mL aliquots up to 1 year at −20°C

First strand synthesis buffer, 5× (Invitrogen Superscript III)

100 µL 1 M Tris-HCl, pH 8.3 (*APPENDIX 2A*; 250 mM final)

150 µL 1 M KCl (375 mM final)

6 µL 1 M $MgCl_2$ (15 mM final)

144 µL deionized water (400 µL total volume)

Store up to 6 months at −20°C

Formamide loading buffer, 2×

940 μL deionized formamide

40 μL 0.5 M EDTA, pH 8.3 (*APPENDIX 2A*; 20 mM final)

20 μL 2% (w/v) xylene cyanol

20 μL 2% (w/v) bromphenol blue

Store up to one year at −20 °C

COMMENTARY

Background Information

X-ray-dependent hydroxyl radical footprinting—Hydroxyl radicals have been widely used to probe the conformation of nucleic acids and nucleic acid–protein complexes (Latham & Cech, 1989; Strahs & Brenowitz, 1994; Tullius & Dombroski, 1986). Reaction of hydroxyl radical with ribose results in oxidation of the sugar and elimination of phosphate groups, leading to strand cleavage.

Cleavage in the presence of hydroxyl radical is relatively insensitive to base sequence and secondary structure of the RNA (Celander & Cech, 1990). However, the susceptibility of individual nucleotides to cleavage in the presence of a hydroxyl radical correlates well with the solvent accessibility of the ribose C4′ (Balasubramanian et al., 1998; Cate et al., 1996; Latham & Cech, 1989). Nucleotides that are inaccessible to bulk solvent due to RNA tertiary structure or interactions with a protein are protected from cleavage. The resulting footprint provides information about the conformation of the nucleic acid, and can be quantified to determine the fraction of structured molecules in the population.

Chemical methods for generating hydroxyl radicals, such as the Fenton-Haber reaction (Dixon et al., 1991) or disproportionation of peroxynitrous acid (King et al., 1993), typically require several seconds or longer to cleave 20% to 30% of the RNA. This unit describes the use of a synchrotron X-ray beam to generate hydroxyl radicals in aqueous solution. The advantage of this method is that cleavage reactions can be completed in 0.1 to 100 msec, even in the presence of radical scavengers. Therefore, “X-ray footprinting” can be used to probe kinetic folding intermediates or other transient conformations.

The protocols given here were initially developed to probe the folding pathway of the *Tetrahymena* ribozyme (Sclavi, Sullivan, et al., 1998), but can be applied to a variety of catalytic RNAs or RNA-protein complexes. For example, we have used this approach to probe ribosome assembly intermediates in vitro (Adilakshmi et al., 2008) and in cells (Clatterbuck Soper et al., 2013).

To carry out cleavage reactions on nucleic acids in the millisecond timescales, the beam must deliver a sufficient flux of photons to the sample so that the steady-state concentration of hydroxyl radicals is at least 0.5 to 1.2 μM (Ralston, Sclavi, et al., 2000). Hydroxyl

radicals are produced after excitation of water molecules by absorbed photons, according to the following equation (Klassen, 1987).

$$H_2O \xrightarrow{hv} H_2O^+ + e^-_{dry} \xrightarrow{H_2O} H_3O^+ + \bullet OH + e^-_{aq}$$

The steady-state concentration of hydroxyl radicals and the dose required to cleave the RNA within the sample depend on many factors, including the flux density of the beam and the energy absorbed by the sample (Bohon et al., 2014). XFP (NSLS II-17BM) delivers a broad spectrum of X-rays (5–16 keV) using a three-pole wiggler (3PW) insertion device. When operating at its maximum of 500 mA, XFP is projected to deliver 3×10^{13} photons/sec into a 5-μL sample near its peak energy of 7.2 keV (3×10^{16} photons/sec from the full spectrum of the beam). Absorption of 10 keV produces 287 hydroxyl radicals (Klassen, 1987). The steady-state concentration of hydroxyl radical can be estimated from the photon flux, and is ~10^{-5} M for NSLS-II XFP. The effective concentration of hydroxyl radicals will be reduced by free radical scavengers in the sample (see Critical Parameters).

Instrumentation—For X-ray footprinting of RNA, the sample must be placed wholly in the path of the beam, so that the entire sample receives as uniform X-ray dose as possible. This is most simply achieved by a steel multi-sample holder (Figure 11.6.1) that can be chilled to −30 °C to prevent frozen samples from thawing (Adilakshmi et al., 2009). Alternatively, the sample can be passed through silica tubing in a flow cell (Bohon et al., 2014; Gupta et al., 2014). In either case, the sample is aligned with the beam using a detector placed behind the sample block. Precise alignment of the sample with the beam is necessary to obtain reproducible RNA cleavage. At NSLS-II XFP, a toroidal focusing mirror (Bohon et al., 2016; Sullivan et al., 2008) is used to regulate the cross-section of the beam from as small as 120 μm x 450 μm, which is suitable for exposing sample in a narrow capillary, to 3 mm x 3 mm, which can be used to expose larger cell pellets. A kilopixel diamond imaging detector provides real-time feedback on the intensity of the beam and its dimensions, which has substantially improved the reproducibility of the results (Bohon et al., 2010; Zhou et al., 2015).

At XFP, typical irradiation times for RNA footprinting are 0.1–1 ms for RNA solutions and 10–100 ms for frozen cells (Hulscher et al., 2016). The exposure time is regulated by an electronic shutter placed between the sample block and the end of the beampipe in the case of the multi-sample exposure apparatus. The path of the beam between the end of the beampipe and the shutter should be short or covered by a retractable flight tube to minimize scattering of the beam before it reaches the sample and to reduce scattered radiation in the experimental hutch. The multi-sample holder is connected to a water bath to maintain constant temperature. This is sufficient to dissipate the small amounts of heat generated over short (<100 msec) irradiation times. Alternatively, a flow cell with a narrow capillary (0.2 to 0.7 mm inner diameter) is mounted in the path of the beam. When connected to a pump, this allows RNA solutions or live culture to be pumped past the beam. In this configuration, the total X-ray dose depends on the intensity of the beam incident on the sample, and on the flow rate which governs the time needed for the sample to pass by the beam.

A modified KinTek® stopped-flow rapid-quench apparatus has been used for rapid mixing experiments (Figure 11.6.3) at the NSLS X28C beamline. The mixing valve present in a standard rapid-quench apparatus is replaced with a flow cell mounted on a steel plate at the back of the box. This enables the flow cell to be placed close to the end of the beampipe (Dhavan et al., 2001). The flow cell itself is constructed of Vespel to minimize damage from X-rays.

Sample and buffer are driven through the lines by syringes at the top of the apparatus. After samples (10 to 20 μL) flow through a mixing T, they are aged in a 60-μL loop before being pushed through the flow cell. If desired, a quench solution can be delivered via a third syringe. However, a chemical quench is not normally necessary, because the hydroxyl radical concentration decreases rapidly (<1 msec) as soon as the beam is turned off (Sclavi, Woodson, et al., 1998).

The KinTek® RQF-3 uses a stepper motor and platform to advance the pistons of the drive syringes. The distance traveled by the sample is determined by the number of turns of the stepper motor. The time required to advance the sample over this distance depends on the motor speed. Typical parameters for RNA footprinting at X28C are given in Table 11.6.1. The exposure time is regulated by controlling the speed at which the sample flows past the exposure port. The exposure time is given by

$$t_{\text{xray}}(\text{msec}) = \frac{(0.04\,\text{rev})}{(s\,\text{rpm})} \times \frac{60\,\text{sec}}{1\,\text{min}} \times 1000$$

Hence, a 10-msec exposure would require a motor speed, *s*, of 0.024 rpm.

RNA analysis—X-ray footprinting patterns may be analyzed by direct electrophoresis of ^{32}P-labeled RNA fragments (Sclavi et al., 1997), or by extending gene-specific primers with reverse transcriptase and sequencing the cDNA on traditional sequencing gels (Inoue & Cech, 1985; Moazed et al., 1986) or by capillary electrophoresis (Shcherbakova et al., 2008). Each primer extension reaction requires 0.5–1 μg RNA and can be expected to give sequence data for roughly 200 nucleotides (traditional sequencing gel) or 300–400 nucleotides when analyzed by capillary electrophoresis. The band intensities in the gel can be analyzed using the Semi-Automated Footprinting Analysis (SAFA) software package (Das et al., 2005) or ShapeFinder (Vasa et al., 2008) package. More recently, high-throughput sequencing methods for analyzing the distribution of RNA cleavage products have been developed (Kielpinski et al., 2015; Kielpinski & Vinther, 2014) (see Unit xx). The advantage of this approach is that it does not require gene-specific priming and can cover several RNAs at one time. A disadvantage is that the library preparation can introduce additional bias and noise into the fragmentation profile that must be carefully controlled during library preparation and analysis.

Critical Parameters

Although synchrotron X-ray footprinting provides sequence-specific information about the tertiary conformation of RNAs that cannot be presently obtained by other methods, several

variables will determine the likelihood of success. The critical parameters discussed below include conformational stability of the RNA, optimum exposure times, factors influencing signal-to-noise ratios of product bands, and sample handling.

Conformational stability—X-ray-dependent footprinting will be successful only if the hydroxyl radical protection pattern produced by the folded RNA or RNA-protein complex is well defined. It is important that the RNA tertiary structure or protein complex of interest is stable under the final conditions of the experiment. If the RNA tertiary structure is unstable, or if the RNA folds into multiple conformations, the footprint will be faint and difficult to interpret. Nonspecific RNA-protein complexes obscure native interactions and hamper interpretation. It is important to optimize the conditions for refolding or RNA-protein assembly before undertaking experiments at the synchrotron beamline. Parameters that should be evaluated include buffer, pH, Mg^{2+} concentration, temperature, protein concentration, and protocols for annealing the RNA. For in-cell footprinting experiments, it is necessary to confirm that the RNA of interest is sufficiently expressed to obtain good cDNA synthesis.

Hydroxyl radicals react with nucleic acid bases as well as the ribose moiety (Breen & Murphy, 1995; Dizdaroglu & Bergtold, 1986). In general, these modifications do not lead to strand scission and are not detected by the protocols described here. The most common base modifications, 8-oxo-G and 5-hydroxy-C, are not expected to drastically destabilize RNA secondary structure and are bypassed by most reverse transcriptases (Wallace, 1998). Nonetheless, it is important to be aware that specific base modifications could induce unfolding of the RNA, and this could alter the observed cleavage pattern. Similarly, rapid oxidation of amino acid side chains may destabilize RNA-protein complexes and cause them to dissociate during the X-ray exposure.

Exposure time—To interpret footprinting experiments quantitatively, it is important that the extent of cleavage be adjusted so that each molecule is cleaved no more than once, on average, within the sequence detection window. For direct detection of labeled RNA fragments or for analysis by cDNA synthesis, this is usually achieved by limiting the extent of cleavage to 10% to 30% of the starting material (Brenowitz et al., 2002). It also depends on the length of the RNA (longer RNAs require shorter exposure times). If the cleavage pattern is to be analyzed by high-throughput sequencing with short reads, the total extent of cleavage must be much higher (10–20% full-length) to ensure adequate signal to noise. Ideally, the proper exposure time should be determined experimentally for each target RNA by acquiring a dose-response curve (Figure 11.6.4) in conditions that are as similar as possible to those that are to be used in the actual experiment (see Support Protocol 2). The exposure time will depend on the flux density of the X-ray beam. If the beam flux changes substantially, the optimal exposure time must be redetermined.

The extent of RNA cleavage will be reduced by the presence of free radical scavengers in the sample, such as Tris, Hepes, thiols, and glycerol. Inorganic buffers such as phosphate or cacodylate give excellent results and should be used when possible. Concentrations <30 mM of Tris can be tolerated, as long as the exposure time is lengthened to compensate for the reduced rate of cleavage. Carboxylic acids (such as EDTA) and urea do not interfere with

hydroxyl radical cleavage (Ralston, He, et al., 2000). Proteins readily react with hydroxyl radical and mixed RNA-protein samples typically require longer X-ray exposures than samples containing only RNA. Frozen samples allow less diffusion of hydroxyl radicals and generally require much longer exposures than liquid samples (Adilakshmi et al., 2009).

Signal-to-noise ratio—The ability to quantify the extent of protection by primer extension will depend on the intensity of the product bands from the treated sample relative to the background activity from the untreated control sample. Several factors influence the signal-to-noise ratio. First, it is essential to use a sufficient amount of RNA, so that individual cDNA bands can be detected using a phosphorescent screen or other imaging device. Since the signal intensity depends on the number of products, less material is required to analyze shorter RNAs. Analysis by capillary electrophoresis, which is suitable for longer RNAs, typically requires 0.5 to 1 pmol per primer extension reaction. CE reads typically cover 300–500 nt, beginning 75–100 nt downstream of the primer.

High-throughput sequencing of RNA fragments relies on the ligation of adaptors to the 3′ end of the cDNA. Because the efficiency of cDNA priming and adaptor ligation reaction depends on the RNA (cDNA) sequence, additional noise is introduced during library construction. The effects of this additional noise can be mitigated by the use of random bar codes (Kielpinski & Vinther, 2014), good sequencing coverage (read depth ≥ 1000 per residue), suppression of unwanted reads (Spitale et al., 2014; Talkish et al., 2014), and proper statistical analysis of the data (Kielpinski et al., 2015).

Another critical parameter for enhancing the signal-to-noise ratio is to minimize background cleavage of the RNA. The initial (unexposed) RNA sample must be very high quality with minimal fragmentation. The samples should be protected from scattered X-radiation when the beam is on. Trace contamination by ribonucleases should be minimized to the extent possible. Besides incautious preparation of buffers and samples, common trouble spots are mishandling of the exit tube and sample-loading syringe on the KinTek® stopped-flow apparatus, prolonged exposure of tips and samples to airborne contaminants, and contaminated O-rings and lids in screw-cap microcentrifuge tubes. Nucleases can be removed by soaking the sample ports and internal tubing for 1 hr or overnight in a detergent such as Absolve (NEN). The detergent must be removed by thorough rinsing with RNase-free water and ethanol. Additional control reactions can be added to the protocol to troubleshoot sources of background cleavage. When footprinting RNA in cells, the cells should be kept frozen and held at −80 C until the RNA can be isolated. We have obtained good results usingTrizol or Qiagen RNA mini-preps to isolate the RNA. The quality of the RNA isolation is critical to the success of the experiment.

Anticipated Results

Quantitative analysis of footprinting experiments has been discussed in detail elsewhere (Hsieh & Brenowitz, 1996). Nucleotides that become protected from hydroxyl radical cleavage because of tertiary structure or protein binding should be visible as clear regions within a ladder of bands on the gel. Comparison with a sequence ladder should permit

assignment of the protected regions to particular sequences within the RNA. Interpretation of the data is improved considerably if a three-dimensional structure or model is available.

The relative extent of protection is determined by comparing the intensity of cleavage products in unfolded and fully folded controls. A detailed presentation of direct integration of RNA or cDNA products can be found in *CPMB UNIT 12.4*. The analysis of high-throughput sequencing of hydroxyl radical footprinting (HRF-Seq) is described in Unit xx (Kielpinski et al., 2015). The equilibrium folding of RNA as a function of Mg^{2+}concentration can be described by the Hill equation. The fractional saturation of a protected region, $\bar{Y}$, is fit to

$$\bar{Y} = \frac{P - P_{\min}}{P_{\max} - P_{\min}} = \frac{(K_{\text{app}}/[\text{Mg}^{2+}])^{n_{\text{H}}}}{1 + (K_{\text{app}}/[\text{Mg}^{2+}])^{n_{\text{H}}}}$$

where K_{app} is an apparent dissociation constant corresponding to the midpoint of the transition, n_{H} is the Hill coefficient, and $P_{\min}$ and $P_{\max}$ are the upper and lower baselines of the transition (see Basic Protocol 3, steps 19 and 20). An example of this type of data is given in Figure 11.6.5.

Time-dependent experiments should be fit to an appropriate rate expression, as illustrated in Figure 11.6.5. For single first-order transitions, this yields

$$\bar{Y} = \frac{P - P_{\min}}{P_{\max} - P_{\min}} = 1 - e^{-k_{\text{obs}}T}$$

The first equality holds only if the upper plateau of the kinetic transition defined by $P_{\max}$ represents the full extent of folding, as determined from equilibrium experiments. Because the progress curves for each nucleotide are normalized to cleavage of that nucleotide at the start and end of the experiment, it is essential to thoroughly establish these endpoints. For example, to measure the assembly kinetics of the 16S rRNA, we used deproteinized 16S rRNA as our starting sample and mature 30S ribosomes as our endpoint control reaction (Adilakshmi et al., 2008). These endpoints were determined in triplicate.

Transitions that occur on the 0.1-sec to 10-sec timescales are easily monitored by the current state of equipment at beamline XFP, which is mainly limited by the mixing time of the rapid quench. Slower transitions (1 to 10 min) can be detected by injecting inducer at a specific time during a flow experiment as in Basic Protocol 2. With the future development of faster mixing devices, it should be possible to probe in vitro RNA folding reactions occurring on timescales of <20 msec, given the high flux density and narrow focus of XFP which allows for very short (~0.1 msec) exposure times.

Time Considerations

Experiments should be planned 2 to 4 months in advance. Preparation of samples for shipment to the beamline requires 1–2 weeks, and samples must be shipped at least 24 hr in advance. Careful planning and execution are essential, as experiments at the beamline are often scheduled over a 1- to 3-day period. Roughly 2 weeks should be allowed for gel electrophoresis and for analysis of the data, or 1 month for the preparation and sequencing of libraries for high throughput analysis.

Acknowledgments

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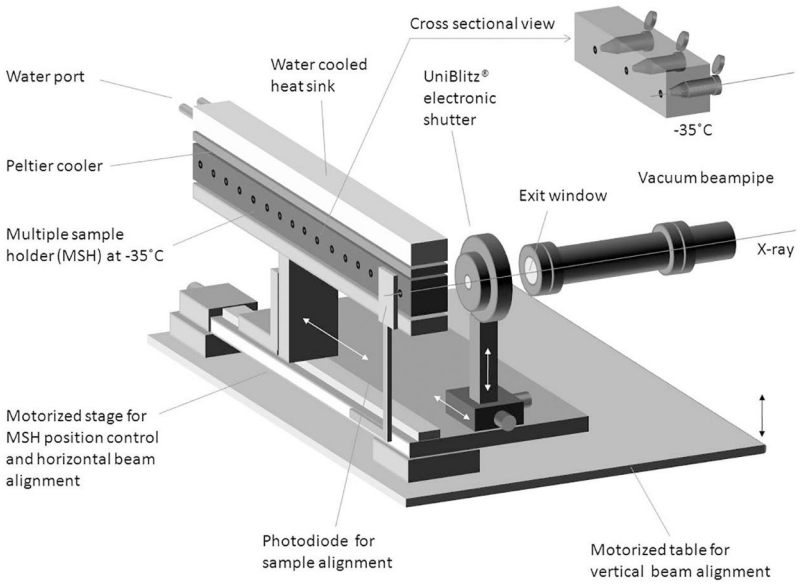

Figure 11.6.1.

Multi-sample holder for equilibrium footprinting experiments. Adapted from Adilakshmi et al. (2009) with permission from Harcourt.

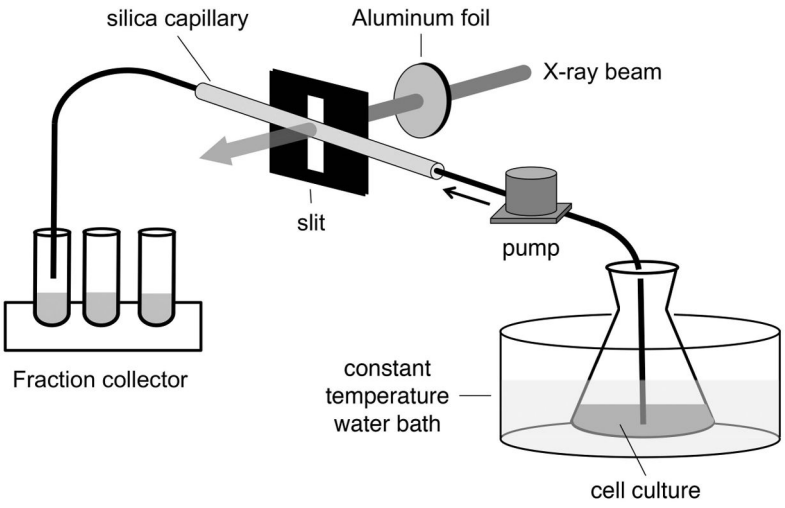

Figure 11.6.2.
Flow set-up for irradiation of live bacterial culture. Adapted from Hulscher et al. (2016) with permission from Harcourt.

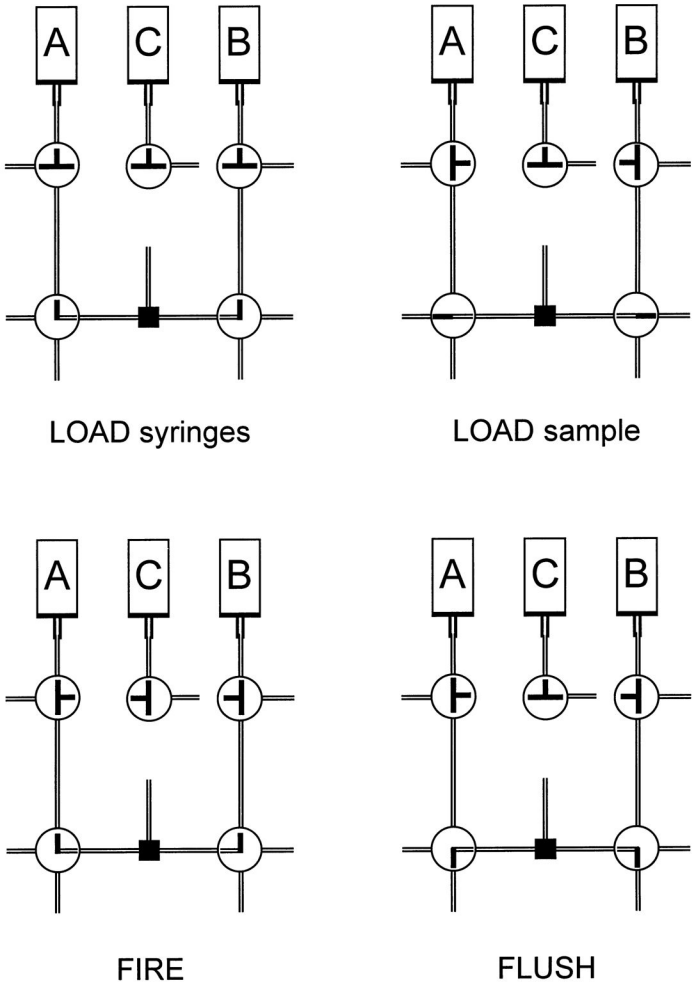

Figure 11.6.3.
Modified rapid-quench apparatus for time-resolved footprinting. Adapted from Sclavi et al. (1998b) with permission from Harcourt.

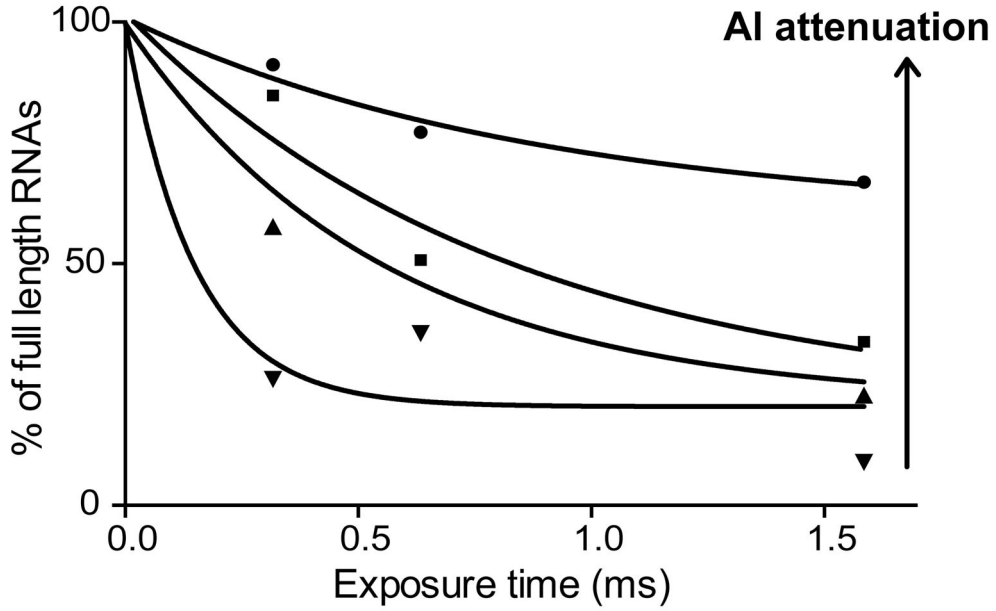

Figure 11.6.4.

Dose-response for cleavage of 16S rRNA in E. coli MRE600 cells at XFP. The beam incident on the sample was attenuated with aluminum of different thickness. Inverted triangle, 152 μm; triangle, 203 μm; square, 305 μm; circle, 508 μm. The flow rate ranged from 1 to 5 mL/min.

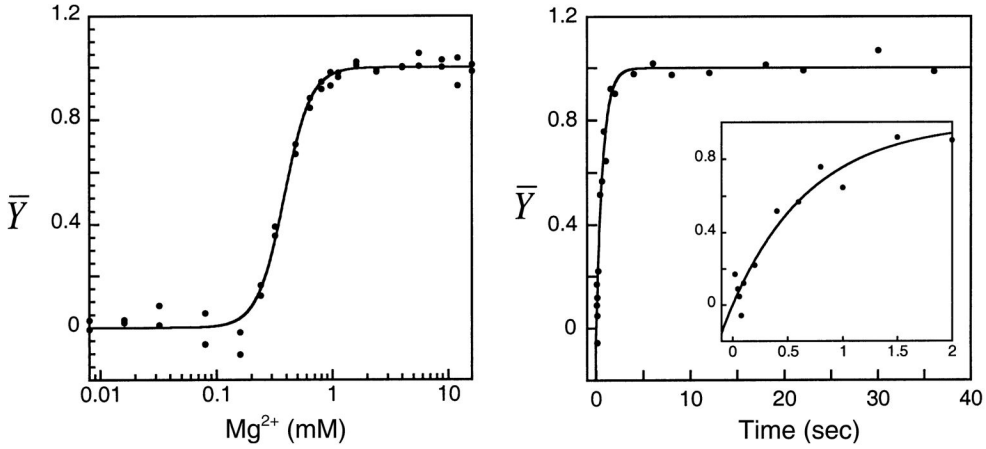

Figure 11.6.5.

Results of X-ray footprinting experiments showing Mg^{2+}-dependent folding of the P4-P6 domain of the *Tetrahymena* ribozyme. Reprinted from Deras et al. (2000) with permission from the American Chemical Society.