

# Semiautomated and rapid quantification of nucleic acid footprinting and structure mapping experiments

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**We have developed protocols for rapidly quantifying the band intensities from nucleic acid chemical mapping gels at single-nucleotide resolution. These protocols are implemented in the software SAFA (semi-automated footprinting analysis) that can be downloaded without charge from <http://safa.stanford.edu>. The protocols implemented in SAFA have five steps: (i) lane identification, (ii) gel rectification, (iii) band assignment, (iv) model fitting and (v) band-intensity normalization. SAFA enables the rapid quantitation of gel images containing thousands of discrete bands, thereby eliminating a bottleneck to the analysis of chemical mapping experiments. An experienced user of the software can quantify a gel image in ~20 min. Although SAFA was developed to analyze hydroxyl radical ( $\cdot\text{OH}$ ) footprints, it effectively quantifies the gel images obtained with other types of chemical mapping probes. We also present a series of tutorial movies that illustrate the best practices and different steps in the SAFA analysis as a supplement to this protocol.**

## INTRODUCTION

Chemical mapping of nucleic acids ('footprinting') provides structural information at nucleotide resolution. Chemical probes provide unique structural detail. As examples, hydroxyl radicals ( $\cdot\text{OH}$ ) probe the solvent accessibility of the nucleotide backbone<sup>1-5</sup> whereas a chemical modifier such as dimethylsulfate can identify exposed base moieties that are base-paired<sup>6,7</sup>. Boundaries of single stranded regions, as well as an estimate of their flexibility, can be obtained by chemical probing using the SHAPE chemistry<sup>8-10</sup>. The importance of chemical mapping to RNA and DNA structural analysis stems from the ease with which nucleic acid polymers are separated by size using gel electrophoresis. Chemical mapping is thereby often used to follow structural change in response to titrated changes in equilibrium solution conditions (such as  $\text{Mg}^{2+}$  concentration)<sup>11-14</sup> or as a function of time<sup>3,15,16</sup>. Such quantitative mapping experiments require the analysis of large numbers of gels. Software tools that assist in the analysis of this cornucopia of information in a standardized manner are thus needed.

**Figure 1** illustrates a gel image based on a typical chemical mapping reaction carried out on a large structured RNA molecule. 'Protections' from the chemical probe are observed with increasing  $\text{Mg}^{2+}$  (the bands get lighter) and are characteristic of the RNA folding. Single-nucleotide band fitting algorithms allow an accurate estimation of the relative intensities of each band in the gel using a deconvolution procedure<sup>17,18</sup>. This deconvolution procedure effectively corrects for errors in observed band intensity that are the result of peak overlap. **Figure 1b** illustrates the result of this deconvolution procedure for one of the lanes in the gel; a series of individual peak models (shown in light gray) are fit to the profile of the lane (black line). The SAFA (semi-automated footprinting analysis) procedure allows a user to quantify a gel image using a single-nucleotide fitting procedure in a semiautomated way<sup>18</sup>. This protocol describes the steps to be taken to obtain quantitative and reproducible data from a gel image using SAFA (**Fig. 1a**).

Single-nucleotide peak-fitting algorithms require initial guesses for the peak positions in each lane<sup>17,18</sup>. Most gels, however, are not sufficiently uniform to easily transfer assignments of bands from one lane to the next. Therefore, we implemented a gel rectification procedure (**Fig. 2**) that allows users to geometrically correct gel images in a standard and semiautomated fashion so that band assignments are uniform across the gel. This procedure enhances the accuracy and reduces the tedium of quantifying all the band intensities of each gel. This procedure also allows SAFA to average the band signal for each lane, resulting in a more robust quantification of the band intensity.

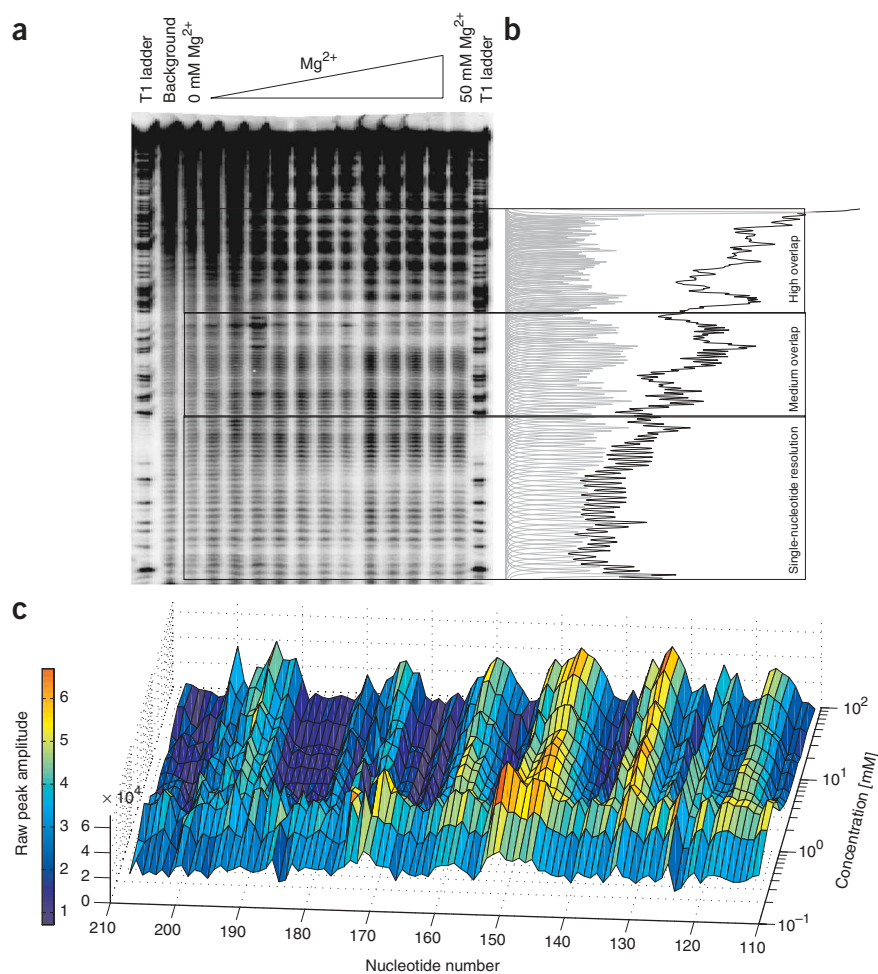
The expected outcome of a SAFA analysis is illustrated in **Figure 1**. A digitized gel image is input into SAFA. SAFA rectifies the lanes and bands to remove geometric distortion (**Fig. 1a**) and fits a peak model (**Fig. 1b**) to accurately quantify the individual peak intensities (**Fig. 1c**)<sup>18</sup>. The procedure is semiautomated so that an experienced user can quantify an entire gel image in ~20 min. Obtaining highly reproducible results requires some experimental planning (discussed below), as the layout of the lanes on the gel can greatly facilitate gel rectification. The inclusion of sample replicates allows the reproducibility of the data to be estimated.

Accurate gel quantitation is a key step in the analysis of chemical mapping reactivity data. In applications to structural modeling, thermodynamic titrations, or time-resolved kinetic experiments, the resolved band intensities must subsequently be normalized postquantitation for subsequent quantitative analysis. We therefore include in this protocol instructions on using the data normalization approaches implemented in SAFA for  $\cdot\text{OH}$  footprinting. A user should be able to efficiently and reproducibly quantify a gel image using the approach outlined below.

Although the example developed in this protocol is based on a thermodynamic analysis of the folding of an RNA molecule using  $\cdot\text{OH}$ , the quantitation methodology is independent of the

**Figure 1** | Basic premise of the SAFA software.

(a) SAFA-rectified chemical mapping gel image for quantitative analysis. In this case, a  $Mg^{2+}$  titration of the L-21 *T. thermophila* group I intron. (b) Peak model (gray line) fit to the profile of the gel (black line) during the quantitation procedure. Regions of low, medium and high overlap are indicated. (c) Expected outcome of the protocol; raw peak amplitudes based on the gel image as a function of concentration and nucleotide number. Using SAFA, a user can quantify the gel image in ~20 min.



experimental modality. Published studies demonstrate the generality of SAFA in structural, thermodynamic and kinetic studies of nucleic acids with a variety of chemical probes<sup>10,19–21</sup>. Structural analyses of nucleic acids include ·OH Footprinting and chemical mapping, which respectively report the solvent accessibility and the extent of base-pairing, at either a rough (DMS mapping) or fine level (SHAPE mapping)<sup>6,22</sup>. These techniques can be used to assess thermodynamic and kinetic parameters for nucleic acid molecules by monitoring changes in the relative protections as a function of a reaction coordinate (e.g., salt concentration) or time<sup>13,15,23,24</sup>. The core algorithms in SAFA are being adapted to analyze high-throughput chemical modification data from capillary sequencers as well 2D nucleic acid gels for mapping contacts in large RNA molecules. In the spirit of the RNA Ontology Consortium<sup>25</sup>, we continue to develop SAFA with the goal of accommodating the needs of the users of the full range of available chemical mapping methods. Beyond an already-facilitated quantification of diverse

nucleic acids probing and mapping experiments, future versions of SAFA will include multiple normalization options such as normalization to one particular band<sup>8</sup> or by pairwise comparison<sup>19</sup>.

## MATERIALS

### EQUIPMENT SETUP

Versions of SAFA have been compiled to run on the Mac OS X (≥10.4, PPC/Intel) and Windows XP operating systems. The different versions of the software, along with the source code can be downloaded gratis from <http://safa.stanford.edu>. Specific installation instructions are provided in the software documentation for the different architectures. SAFA has been tested on a variety of platforms; we recommend a system with at least 2 GB of RAM and a recent Pentium processor. A three-button mouse is also useful for both Macintosh and PC versions, but not required.

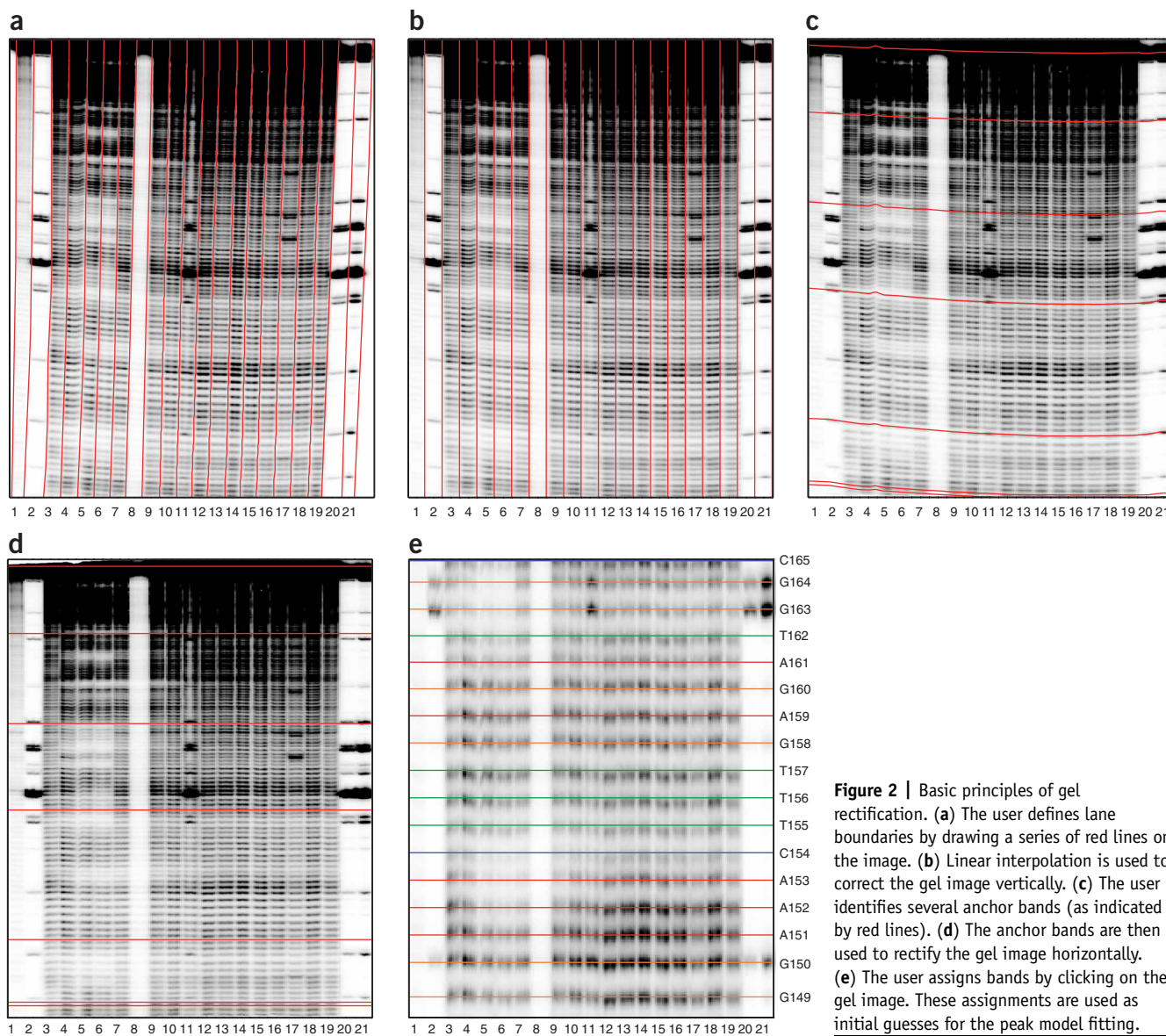
## PROCEDURE

### Preanalysis experimental planning

**1** | Run the gel referring to the following layout guidelines. Careful layout of the lanes in the gel improves the accuracy of the resolved peak densities due to the nature of gel rectification. **Figure 3** illustrates the recommended layout of a gel. Three types of lanes are expected: (i) *Sequencing lanes*: These lanes guide band assignment. They generally have a dark band at a particular residue type. For example, an RNA T1 digest will produce a dark band for every G in the sequence. Running sequencing reaction lanes for more than one nucleotide may be necessary for mapping methods such as SHAPE<sup>10</sup>; (ii) *Background lanes*: These lanes contain unmodified RNA and help estimate the amount of background cleavage that has occurred during the chemical mapping experiment; and (iii) *Data lanes*: These lanes contain the experimental data. In the case of a titration, each lane corresponds to the profile for a particular concentration of solute in the series of concentrations. In other experiments, these may contain pairwise comparisons of the mapping in presence or absence of an influencing solute or thermodynamic variable. We strongly recommend that experiments be replicated, ideally with independently prepared samples, to assess the error in the final quantitated values.

SAFA reads as input either ImageQuant (Molecular Dynamics) .gel files or standard .tiff images. When using SAFA in conjunction with image analysis software other than ImageQuant, it is important to obtain the highest bit-depth possible (e.g., 16-bit images should generally be used). See Step 1 for guidelines regarding the layout of the gel.

SAFA also requires a nucleotide-sequence file in FASTA format that corresponds to the sequence of the nucleic acid polymer being studied. Example files are available for download at <http://safa.stanford.edu>.



**Figure 2** | Basic principles of gel rectification. (a) The user defines lane boundaries by drawing a series of red lines on the image. (b) Linear interpolation is used to correct the gel image vertically. (c) The user identifies several anchor bands (as indicated by red lines). (d) The anchor bands are then used to rectify the gel image horizontally. (e) The user assigns bands by clicking on the gel image. These assignments are used as initial guesses for the peak model fitting.

### Importing the gel image and sequence files

2| Open SAFA by clicking on its icon or by typing 'SAFA' in the command line window of Matlab. The sequences and gel files are imported by clicking on the 'Load Sequence' and 'Load Gel' file buttons on the main SAFA window (**Fig. 4a**), respectively (**Supplementary Video S1** online).

3| Define 'crop' boundaries immediately after loading the gel image by left clicking and dragging in the main window. To quantify independently several portions of the same gel image (e.g., samples loaded at different times), the user will need to upload the image as many times as there are sections to be quantified.

4| Import the sequence file by clicking on the 'Load Sequence' button. Sequences no longer than 500 bases should be loaded. Therefore, the sequence file should slightly exceed the number of bases that are expected in the gel image. The numbering assigned to the input sequence can subsequently be adjusted to match the conventional numbering used in the literature for the molecule. The user specifies whether the gel involves 5' or 3' labeled nucleic acid fragments, i.e., whether the fragments with faster electrophoretic mobility correspond to smaller or larger residue numbers, respectively.

▲ **CRITICAL STEP** Once the sequence is loaded, a Sequence Dialog appears (**Fig. 4b**). This Sequence Dialog defines the behavior of the semiautomated band assignments. Selecting a base (A, C, G, U or T) will highlight it on the sequence that is displayed. It is recommended to select the nucleotides in the sequence that correspond to bands in the ladder or sequencing lane(s). Therefore, if a

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**Figure 3** | Optimum layout for a gel to be quantified by SAFA. This layout complements the SAFA algorithms and thus yields highly reproducible results. Note that the gel shown in **Figure 1a** has this layout but has been cropped to show only a single titration. This layout allows users to run two experiments on a single gel and then analyze them independently.

T1 digest is used, 'G' should be selected. First time users may want to select all bases, which will require them to manually select each band until they become comfortable with the software.

### Gel rectification

**5** | Identify lane boundaries by clicking on the 'Define Lanes' button in the SAFA main window (**Fig. 4a**). Draw the left most lane boundary on the gel image by left clicking (the middle button and right button undo and terminate the boundary). After defining the first lane boundary, the user may revise the boundary by typing 'r'. After selecting at least two lanes, typing 'g' will generate and show the programs 'guess' for the next lane boundary. **Figure 2a** shows a completed lane boundary selection on a typical gel image. Typing 'Z' will vertically rectify the gel image as illustrated in **Figure 2b**. SAFA allows the user to export the rectified gel image as a tiff for archival purposes (**Supplementary Video S2** online).

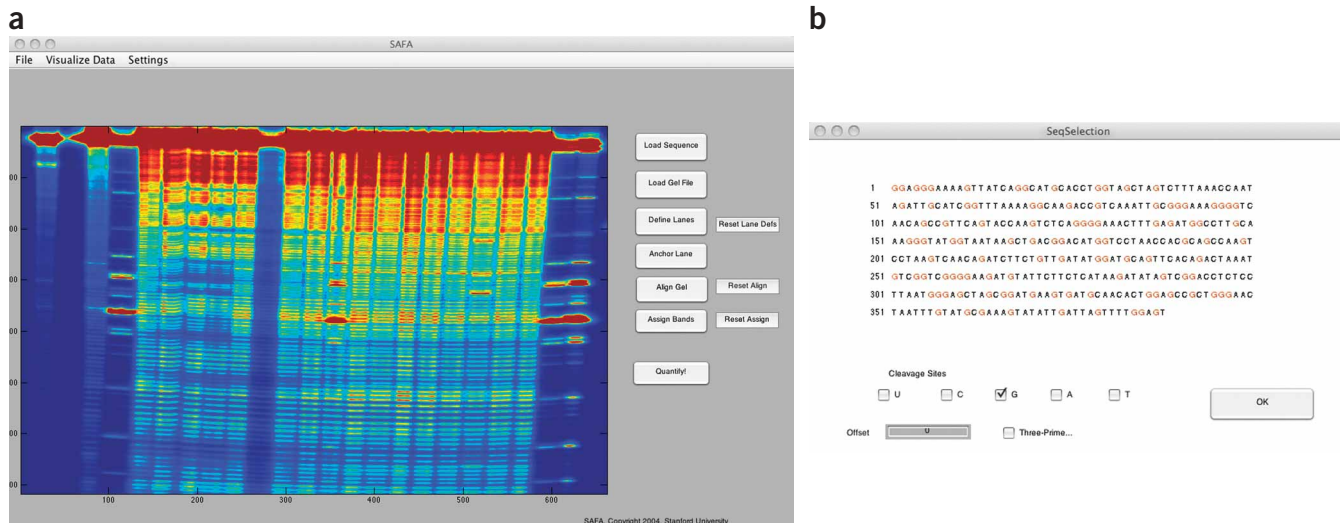
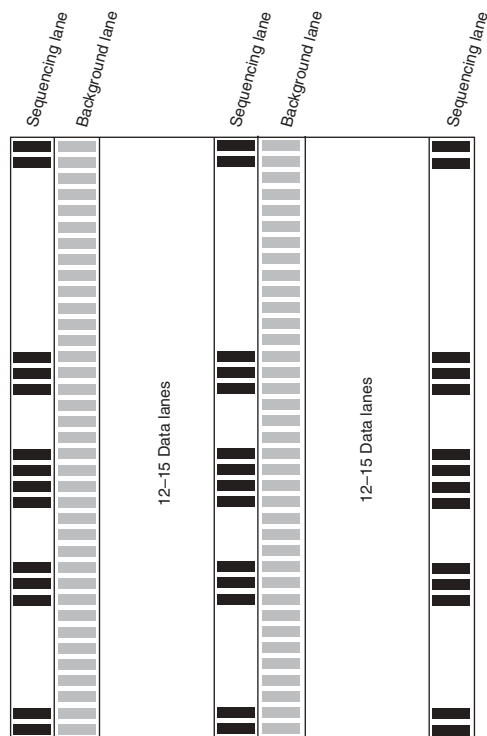
**6** | Click the 'Anchor Lane' button to define the reference lane for horizontal rectification of the gel. The user should select a central lane in the gel for this purpose.

**7** | Click on the 'Align Gel' dialog to define band anchor lines spanning the horizontal width of the gel as shown in **Figure 2c**. Three to four anchor lines distributed evenly over the length of the gel are typically sufficient to rectify the image (**Fig. 2d**). More lines may be necessary for severely or nonuniformly deformed gels (**Supplementary Video S3** online).

**▲ CRITICAL STEP** Zooming in and out of the gel image during anchor line definition by right clicking on the mouse facilitates accurate gel rectification. Furthermore, during all procedures, typing 'c' toggles the gel representation between grayscale and pseudocolor representations.

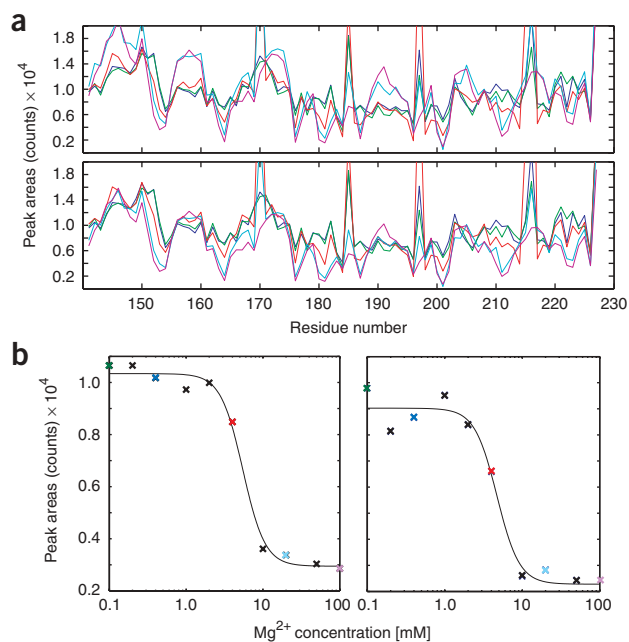
**8** | Save the rectified gel image in .tiff format using the File → Save Image dialog box. It is also prudent to save the results acquired to this point by using the File → Save Dump option.

**▲ CRITICAL STEP** Saving your results periodically is very highly recommended.



**Figure 4** | Screenshot of the SAFA software and identification of the major software features. **(a)** SAFA main window dialog box. The analysis procedure is carried out by clicking on the different buttons on the right hand side of the window as described in this protocol. **(b)** Sequence Dialog defines the behavior of the band assignment feature of the software.

**Figure 5** | Normalization scheme (Invariant Residue Normalization) applied to a  $Mg^{2+}$  titration of the P4-P6 subdomain of the *T. thermophila* group I intron. In this case four invariant residues were used to normalize the data (146, 147, 148 and 195) determined according to ref. 17. **(a)** (Top pane) Raw peak areas as determined by SAFA for lanes corresponding to  $Mg^{2+}$  concentrations of 0.1, 0.3, 6, 11 and 100 mM (green, blue, red, cyan and magenta, respectively). (Bottom pane) Data normalized using invariant residue normalization procedure. A reference lane (in this case the green data (0.1 mM  $Mg^{2+}$ )) is used and then a scaling factor is computed for all the other data such that the differences of the invariant peaks are minimized. **(b)** Titration curves for Residue 182 and 213 based on the data in **a** showing the  $Mg^{2+}$  dependence of these two protections. A Hill model is fit to the data with a cooperativity value ( $n = 3$ ) to obtain a midpoint of 1.7 and 1.9 mM  $Mg^{2+}$  for residues 182 and 213, respectively.



## Band assignment

**9** | Click 'Assign Bands' to start the band assignment procedure. Band assignment provides SAFA with the initial guesses required for fitting a peak model to each lane profile. Assign the bands from the bottom of the gel to the top. The user is asked to input a starting band number (labels on the top of the gel in the SAFA window) and will then click on the gel image at the selected initial band position (**Fig. 2e**). SAFA highlights bands by a colored line (one color per base type). Select the positions for the bases moving up the gel.

**▲ CRITICAL STEP** Although SAFA does not limit the user's access to the bands at the top of the gel, the program is far more likely to yield erroneous results for these poorly separated bands. Limits to the extent of peak-fitting have not been coded into SAFA because gels vary widely in quality and we do not wish to prevent users from extracting all possible information from their gels. Users are advised to use common sense and to not try to overinterpret individual gels. Multiple gels run for increasingly longer times remain essential to accurately quantifying the band intensities of long fragments.

## Quantitation

**10** | Click 'Quantify' to start the fully automated quantification procedure that determines the density of each band. Following quantification, the user should save the spreadsheet summarizing the results in a tab-delimited text file. The first column in the spreadsheet is the nucleotide numbers with the following columns containing the raw peak density data. The user may use this spreadsheet to analyze their data as they see fit; it may be inspected, graphed or output to another program. Step 11 describes the numerical transformations included in SAFA typically conducted for equilibrium and time-resolved chemical mapping experiments (**Supplementary Videos S4** and **S5** online). Other normalization procedures are available through separate modules that will be incorporated into future versions of SAFA (<https://simtk.org/home/normalize>).

## Postquantification analysis

**11** | The raw band intensities generated in Step 5 may be normalized to correct for variation in the amount of sample loaded onto the gel or small variations in extent of the chemical mapping reaction among the samples. Choose a normalization strategy based on the nature of the experiment and the ultimate goal of the analysis. Alternative strategies below that can be accomplished within SAFA are accessed through the Visualize Data → Normalize/Colorplot menu option. These transformations can also be carried out in a spreadsheet program. The options are (A) Invariant Residue Normalization and (B) Mean Protection Normalization (**Supplementary Video S6** online).

### (A) Invariant residue normalization

(i) Choose this option for titration and time-resolved experiments where the goal is to observe a change in the protection value of single nucleotides as a function of a specific variable<sup>17</sup>. This approach yields *relative* changes in protection and is ideal if the goal is to obtain a midpoint of a thermodynamic titration or a rate constant of a time-dependent process. We illustrate an example of this procedure in **Figure 5**.

(ii) The protocol identifies a specified fraction (generally 5%) of 'invariant residues' whose densities do not *systematically* change over the course of the experiment. These are the bands that show the minimum systematic change in protection in the experiment. Type the 't' option in the Normalization dialog box to select the residues chosen by SAFA. The raw peak intensities are then divided by the mean intensity of these invariant residues by SAFA.

### (B) Mean protection normalization

(i) Choose this option to estimate the degree of protection. In the case of ·OH footprinting, the degree of protection is correlated to the accessible surface area of the RNA<sup>26</sup>. This simple procedure works particularly well for experiments where

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the average protection in a reference lane probing unstructured RNA is < 10% of the data lanes. In this case, the mean protection value is computed and all peak amplitudes are divided by it. This approach is particularly useful for comparing protections among different molecules.

### ? TROUBLESHOOTING

#### ● TIMING

Preanalysis experimental setup: The time required to set up an experiment is highly variable and depends on the experimental protocol and the user's experience in the particular mapping technique. Prior to the collection of data, several benchmark experiments should be carried out to assess the reproducibility of the quantified data.

Importing the gel image and sequence files: 2 min. A sequence file is reusable and thus must only be created once.

Gel rectification: 15–30 min, depending on the number of lanes and the experience of the user.

Band assignment: 10–20 min. The time taken to assign bands is dependent on the choices made in the sequence dialog.

This step is accelerated if the user is familiar with the pattern in the sequencing lane(s), either by previous annotation on a printout of the gel or by assigning bands in previous gels with the same nucleic acid sequence.

Quantitation: SAFA takes about 30 s per lane to optimize the peak model on a Pentium Processor with 1 GB of RAM.

Normalization: 1–2 min depending on protocol.

The total time to quantify a gel is in general 20 min.

### ? TROUBLESHOOTING

Troubleshooting advice can be found in **Table 1**.

**TABLE 1** | Troubleshooting table.

Problem	Cause	Solution
The gel file is open but is black	The gel file is not located in the appropriate directory	Relocate the file under the directory from which you launch SAFA
The gel file is open but is not clear and or black	The bit-depth of the image is not suitable for the SAFA display	Change the bit-depth of the image in the software used to save the image or in image editing programs such as Adobe Photoshop  Toggle the visualization mode under the Settings → Render SQRT image
SAFA does not properly guess the lanes when pressing 'g'	The contrast between the two lanes and between the bands in these lanes is either too weak or too strong	Manually define the boundaries for these lanes
The quality of the image decreases after I clicked on 'Anchor lane'	At this point, the gel image is simplified for analysis; this is normal	Increase the bins per lane option when clicking on 'Align Gel'
After I saved my aligned gel, it switches back to its unaligned state	Known bug	SAFA needs to be reset (under File/Reset Application) or restarted after each assignment, or when loading new data
SAFA asks me to assign bands in the wrong order	SAFA has two options to assign bands: from 5' to 3' or from 3' to 5'	Make sure the box 'Three-Prime label' in the Sequence window is unselected if you assign from 5' to 3' (and <i>vice versa</i> for 3' to 5')
When pressing 'Z' after having assigned all the bands, SAFA does not count all the bands	Known bug	Restart SAFA and reassign the bands from the saved aligned gel file
After checking the text file output from SAFA, some peaks don't match with the bands on the gel	Some bands were misassigned (e.g., on the top of the gel or in low contrast regions)	Open the saved assigned gel file and edit bands using the 'E' and 'R' keys. However, you may need to run your samples on a different gel

#### Additional troubleshooting guidelines

Installation and compatibility issues are discussed in detail in the SAFA user manual (<http://safa.stanford.edu>). Due to the wide variety of image formats available, most user inquiries reflect difficulty with obtaining sufficient contrast in the main image window (**Fig. 4a**). This problem typically results from a mismatch of the bit-depth of the image. Users can correct this problem by toggling the visualization mode under the Settings → Render SQRT image. Alternatively, the users can change the bit-depth of the image in the software used to save the image.

A clear understanding of the relationship between the sequence file and the band assignment procedure (Step 4) allows SAFA to be used for a variety of applications. We provide a generic sequence file for SAFA (containing only As) in the accompanying data that can be used to evaluate the utility of SAFA for particular applications. The file can be imported and only A is selected in the Sequence Dialog box, allowing the user to select bands sequentially.

The approach chosen for normalization greatly influences the results. We have found that Mean Normalization is generally best for most applications. Invariant Normalization generally is more reproducible and less sensitive to nuclease peaks in the data. However, the results of this approach are critically dependent on the choice of invariants. For well-characterized RNAs with well defined invariant residues (e.g., the L-21 *T. thermophila* group I intron<sup>14,23,27</sup>) Invariant Normalization is advantageous.

Although SAFA can correct many experimental imperfections in a gel image, the resultant data will only be as good as the quality of the underlying experiment and image. For example, nuclease contamination will cause dark if not saturated bands that can bias the data analysis, especially normalization. Experimental effort should be expended in the design of experiments compatible with SAFA analysis and their careful and clean execution. Overexposure of the images (and therefore saturation of the density signal) must be avoided. Higher variability is generally expected in the upper regions of the gel where the peaks are more overlapping. The fact that gel quantitation requires minutes to complete allows users to freely repeat their analysis and establish the reproducibility of their results.

### ANTICIPATED RESULTS

We provide in the Supplementary materials a SAFA output file (**Supplementary Data** online). The file is a matrix of numbers with the first column having the nucleotide number and the following columns the data for each lane. We also provide normalized results using both normalization techniques outlined in Step 11. In **Figure 5** we show typical results obtained for a thermodynamic folding experiment of a large RNA. In the top pane, the normalized ·OH profiles are shown for various Mg<sup>2+</sup> concentrations, which can then be used to obtain thermodynamic parameters for the folding reaction (in this case the Mg<sup>2+</sup> midpoint).

Note: Supplementary information is available via the HTML version of this article.

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