Measuring Non-Equilibrium RNA Dynamics One Molecule at a Time with SU-8 Microfluidics

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Primary CNF Tools Used: ABM contact aligner, class 2 resist room, VersaLaser, Heidelberg mask writer (2000)

Abstract:

Here we report the design, fabrication and implementation of an SU-8 based microfluidic mixing device to enable non-equilibrium single-molecule fluorescence measurements. The device allows rapid initiation of biological reactions, and, in a subsequent region, probing with fluorescence microscopy at times from 10ms-5s after mixing is complete. This device is used to probe the conformational dynamics of regulatory RNA molecules as they sense and bind their target and non-target ligands.

Summary of Research:

Non-equilibrium measurements are powerful tools to study biological interactions [1]. The rapid initiation of biological reactions and examination of their evolution in time, exposes information that is hidden in simple equilibrium experiments. This technique provides details on short lived intermediates, folding pathways and transition states.

Whilst the benefits of non-equilibrium measurements are numerous, such techniques are rarely applied when compared to their equilibrium counterparts. The major bottle-neck is the lack of commercially available systems to perform these experiments. Only the stopped-flow technique is widely used, but requires high sample volumes, limiting the number of kinetic measurements that have been made. Additionally, in cases where kinetic measurements are applied, the experiments report bulk averages. Therefore, sparse or short-lived intermediates are challenging to identify, and careful analysis is required to resolve the presence of intermediate states along reaction pathways [2].

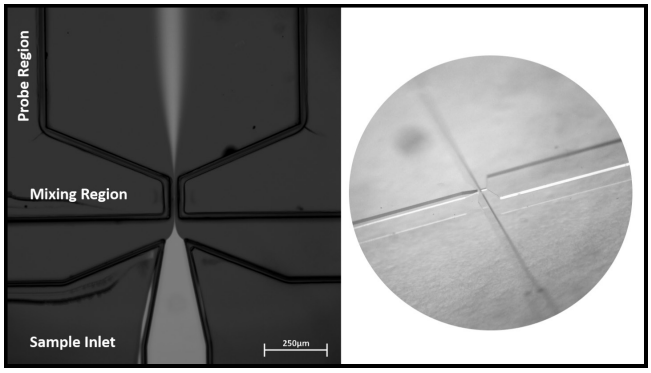

Figure 1: Two images of the SU-8 microfluidic mixing device are shown. At left, a fluorescence image is shown. Fluorescent dye (light grey), enters the sample inlet, and is compressed by flanking buffer streams through the mixing region. Here, the sample stream thins to the micron length scale, allowing rapid diffusion into the sample from the side streams. After mixing, the flow is expanded to allow probing (flow direction bottom to top). The right image shows a stereoscope image of a functional device.

Coupling microfluidic mixing to single-molecule fluorescence circumvents the above challenges. Examination of single molecules allows resolution of individual subpopulations within a sample, nanomolar concentrations are used, and ~millisecond timescales are accessible using microfluidics, with sparse sample consumption [3]. We previously designed and fabricated microfluidic mixing devices to perform such measurements (Figure 1) applying photolithography to pattern a hard material, SU-8.

In brief, a 100 μm thick layer of SU-8 2050 is spun on borofloat, after which it is baked, cured with the ABM contact aligner and developed to yield fluidic channels. A thin sealing layer of SU-8 2005 is next deposited over the top of the fluidic channels, and standard 170 μm

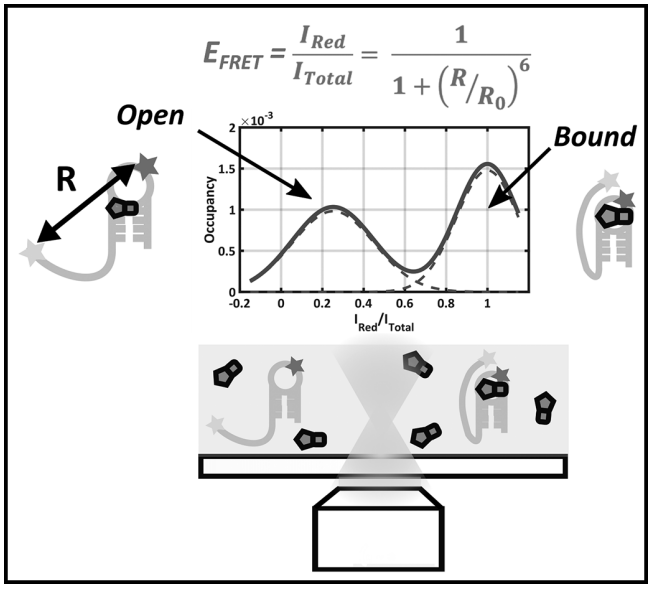

Figure 2: Single-molecule fluorescence measurements use a pair of dyes (represented as stars) to enable FRET. The relative emission of the red dye to the total emission of the dye pair is dependent on the inter-dye distance (R). When suitable positions are found for the dyes, different conformational states of RNA can be distinguished (left and right cartoons). Bound states of regulatory RNAs can be identified (in this case) by EFRET values of 1.

thick glass cover slides attached to produce a stack. The stack is baked, and the sealing SU-8 layer cured. Development removes residual SU-8 that may have entered the channels. The result is a sealed microfluidic device consisting of channel geometries defined by SU-8 sandwiched between two glass layers. The use of SU-8 significantly improves device lifetime and chemical compatibility over traditional soft polymer approaches, allowing extended experiment time with a single device.

With this device we follow regulatory ribonucleic acids (RNAs) as they bind their ligand partners. These RNA elements are labelled with two fluorescent dyes of differing colors, green and red, that form a FRET pair (Figure 2). We excite the green dye with green laser light, and measure the fluorescence emission from the red dye as a ratio of the total emission (sum of green and red dye emission), which yields the FRET efficiency value EFRET. The red emission depends on the inter-dye distance, with smaller inter-dye distances yielding EFRET values that are closer to 1. Probing single-molecules at a time, a distribution of inter-dye distances (EFRET) can be built that resolves subpopulations within our RNA sample. When bound to their ligand partners, a value close to one is expected; unbound states have larger inter-dye distances, and thus a lower EFRET value.

Pairing our microfluidic mixing device with single-molecule fluorescence measurements, we can rapidly introduce different ligand partners to our RNA sample, and watch the conformational transitions at varying times after mixing is complete. The resulting picture illustrates transitions between conformational states in the sample, and furthermore, provides quantitative measurement of rates and bound fractions for differing conditions. The results (Figure 3), show differences in the rates between two ligand partners for this particular RNA. Binding of the target (native) PreQ1 ligand occurs in ~ 500 ms, whilst the off-pathway Guanine binds in ~ 5000 ms.

The conformational re-arrangements required to transition from the unbound to the bound state are much more energetically favorable in the case of PreQ1 than for Guanine. The small differences in chemical composition of these two partners speaks to the highly evolved specificity of this RNA for its native ligand [4].

References:

[1] Al-Hashimi, H. M.; Walter, N. G. Curr. Opin. Struct. Biol. 2008, 18 (3), 321-329.

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[4] Plumridge, A.; Pollack, L. In preparation 2019.

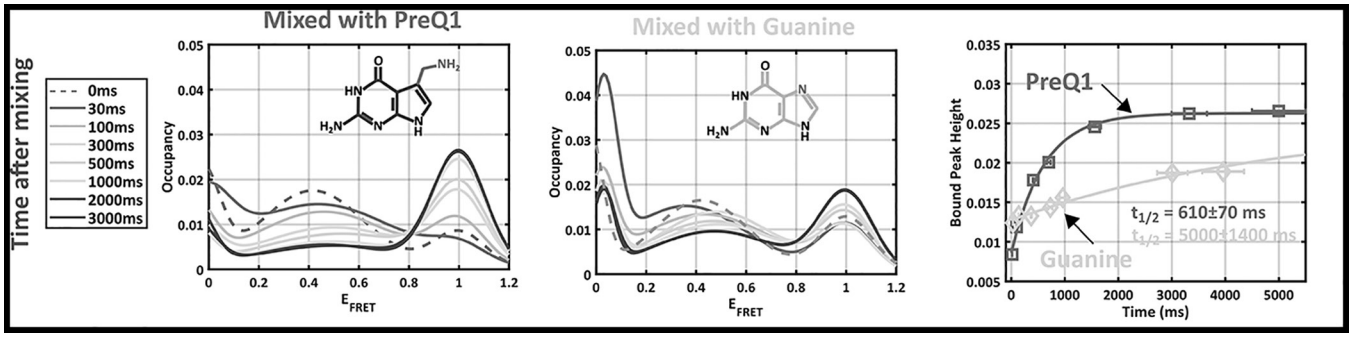

Figure 3: Pairing microfluidic mixing with single-molecule fluorescence allows us to observe RNA transitions from open to bound states at various times after mixing with ligand. In this case we probe transitions after mixing the on path (native) PreQ1, and the off path Guanine (non-native) ligands. Plotting the bound peak height as a function of time after mixing (far right) shows large differences in rates for this conformational change, despite the similar structure of these two ligands (chemical structures shown inset left panels)