**HiP-FA / Supplementary Software**

The HiP-FA software is written in *Labview 9.0*. Open the “HiP-FA Software” script which appears on the top-level of the “HiP-FA\_Labview9.0” library. The interface consists of six different modules: FILE, SETTINGS, ROIs, DNA CONCENTRATIONS, and TITRATION CURVES.

**FILE**. After starting the program, two different file paths are expected: first the raw data which are the raw images acquired with the camera converted into individual ASCII files. For example, the file name “044\_I49\_z11\_ch00.txt”, where “044” is the well number, “I49” the 49th cycle of acquisition, and “Z11” the z-slice number. Select the file corresponding to the very last image acquired i.e. highest well, cycle and z-slice numbers (“044\_I49\_z11\_ch00.txt” in the “Sequence” folder of the exemplary dataset provided. “20140703\_pdm2\_Robot\_Matrix\_dupl1”). Select then a second file containing the numbers of the wells containing the Nile Blue (NB) intercalating dye embedded in agarose which serves as reference to determine the DNA concentrations c(z,t). It should be a text file containing the list of the wells (see “Calibration\_Wells.txt”). The loading of the data can be monitored in the FILE panel. The data, consisting of the two polarization components, can be flipped vertically if necessary by activating the “Flip data” switch. The loading of the data can be deactivated after re-starting the scriSpt to save time by switching the “Load Data“ button to OFF.

**SETTINGS**. The SETTINGS panel is used to set the G factor (gfactor), the concentration of the labelled-DNA reference oligomer (Conc. DNA) , the KD of this sequence (KD1, as determined by the titration of the same sequence as competitor DNA). In addition, the user can specify from which cycle number the data should be analyzed (Cycle Start), the number of cycles and of slices per z-stack to be analyzed (Nr Cylcles and Nr slices, respectively), if it is desirable to analyze only a sub-fraction of the data (most of the information is contained in the firsts 10-20 cycles). “X(raw)” and “z” are used to change the well number and the z-height for the display in panels TITRATION CURVES and DNA concentrations, respectively (see Fig. 1c and Supplementary Figure 2c of the publication).

**ROIs**. The HiP-FA setup is very robust against small misalignments of the optical paths. However, after a new adjustment of the setup it is necessary to specify the two regions of interest (ROIs) corresponding on the raw data to the parallel and perpendicularly polarized components of the emitted fluorescence light (Supplementary Fig. 1b). In this example the parallel component is on top of the image and the perpendicular one on the bottom, but this could be readily inverted by switching “Flip data” to ON in the FILE panel. The two signals appear as Gaussian shaped patterns and the ROIs boundaries (in pixels) of two regions containing the Gaussian patterns can be defined the corresponding commands on the right hand side of the panel. Note that the choice of the ROIs does not need to be accurate since the HiP-FA method is insensitive to slight shifts of FA resulting from the choice of slightly different ROIs.

**DNA CONCENTRATIONS**. The accuracy of the method depends strongly of how well the DNA concentration c(z,t) is determined be using the reference wells containing NB. The software first uses the calibration curve obtained from a direct titration of NB with a competitor DNA sequence (Supplementary Fig. 2a) to compute the competitor DNA concentration c(z,t) for each reference well (thin colored lines in the figure of the panel; the averages of the individual curved are displayed as thick white points). The fitted parameter obtained by using the Hill Equation (Vmax, k, n and y0; see Supplementary Fig. 2a) have to be entered if the “Parameters Hill equation” part of the panel. Due to the relatively low affinity of NB to DNA (~2µM), c(z,t) can be determined accurately only for c>~100nM. The concentrations bellow are extrapolated by fitting the averaged curve using a one-dimensional diffusion model (thick white line; see Methods). This fitting procedure for each z-slice (change “z” in panel SETTINGS for visualization) is critical for the proper construction of the titration curves. To optimize the fitting procedure, it is useful to apply different thresholds for the FA (“Tresh” in “Tresholds”), to fix the offset for the FA values of NB (“offset FA” as the minimum FA value for NB), or to limit the number of cycles to be considered for the fitting procedure (“Cycle End”). Some guess constants can be changed, but this is usually not necessary. Only the parameter “t0” need to be adjusted every time: this parameter corresponds to the time period (in cycles) between the production of the gel matrix and the actual beginning of the measurements (usually this should be < 10-20min in time). Once the quality of the fits are satisfying to each z-height, the DNA concentrations are defined even at low DNA concentrations and the titration curves can be analyzed.

**TITRATION CURVES.** One can visualize the individual titration curves using the “X (raw)” command of the SETTINGS panel. The fitting of the curved is achieved automatically online (in red) according to the procedure described in Methods. Two modes of fitting are possible: by default, all parameters are left free to evolve (see Methods). The concentration of the active protein (Rt) and the dissociation constant (KD2) are displayed in red in the “Fitting parameters” part of the panel. For more robustness and given that usually the protein concentration is the same in every well, the concentration of the active protein can be fixed by switching the “Fix conc. Rt0” to ON and its value can be set by using the command “Rt0”. The parameter B (see Methods) which corresponds to the lower offset of the titration curves is an additional free parameter that can be easily fixed and its value can be set by using the command “Offset”. The value usually corresponds to the FA of the unbound labelled reference DNA oligomer.

The results can be automatically exported by pressing the command “Export” of the TITRATION CURVES PANEL. The software creates a text file containing an ordered list of the fit parameters for all the measurement wells of the plate.

*HiP-FA software / screenshot*

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