Electronic Supplementary Material (ESI) for ChemComm.

Supporting Information

Studying structure and dynamics of self-assembled peptide nanostructures using fluorescence and super resolution microscopy

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Materials. Cyanine5 carboxylic acid (Cy5) and Cyanine3 carboxylic acid (Cy3) were purchased from Lumiprobe (Hallandale Beach, Florida, USA), diphenylalanine (FF) from Bachem (Bubendorf, Switzerland), Fmoc-*L*-Phe-OH from GL Biochem (Shanghai, China), O-(1H-benzotriazole-1-yl)-N,N,N',N'-tetramethyluronium hexafluorophosphate (HBTU) and N-hydroxybenzotriazole anhydrous (HOBt) from Chem-Impex International (Wood Dale, IL, USA), N,N'-diisopropylethylamine (DIEA), triisopropylsilane (TIS), and hexafluoroisopropanol (HFIP) from Sigma Aldrich (Rehovot, Israel), Fmoc-*L*-Phe-Wang resin from Merck (Darmstadt, Germany), trifluoroacetic acid (TFA), dichloromethane (DCM) and N,N-dimethylformamide (DMF) of peptide synthesis purity grade from Bio-Lab (Jerusalem, Israel). All materials were used as received without further purification. Water used was processed by a Millipore purification system (Darmstadt, Germany) with a minimum resistivity of 18.2 MΩ cm.

Probe Peptides Synthesis. The probe peptides (Cy5-FF, Cy3-FF) were synthesized on a CEM Liberty 1 microwave peptide synthesizer (Matthews, NC, USA) using the standard Fmoc solid-phase synthesis strategy. After deprotection of the Wang resin with 20% piperidine and 0.1 M HOBt in DMF solution, Fmoc-*L*-Phe-OH was introduced, followed by introducing the fluorescent dyes. The carboxylic groups were activated by treatment with HBTU/HOBt/DIEA, transforming the carboxylic acids into activated esters to react with the deprotected α-amine groups. After synthesis, cleavage from the resin was performed using a mixture of TFA, TIS and H_2O at a ratio of 95:2.5:2.5. The cleavage mixture and subsequent DCM washing solution were then purged with nitrogen.

The obtained concentrated solution was added to water and lyophilized, and then subjected to reverse-phase HPLC and mass spectrometry for analysis, showing that the product purity was > 95 % (Figure S1 and S2).

Sample Solutions Preparation. For FF alone self-assembly solution, FF dipeptide was first dissolved in HFIP to prepare a stock solution at a concentration of 100.0 mg mL^{-1}. Then, 20 μL of a fresh HFIP stock solution were mixed with 980 μL of water to form a 2.0 mg mL^{-1} 1 mL sample solution. HFIP was added to avoid pre-organization of the solutes.

For epifluorescence microscopy, FF at 100.0 mg mL^{-1} in HFIP and Cy3-FF at 10.0 mg mL^{-1} in HFIP were injected in water to a final concentration of 2.0 mg mL^{-1} and 2.0 μg mL^{-1} (0.1% Cy3-FF), respectively, in a well of a 8-well Labtek chamber.

For PAINT microscopy, FF at 100.0 mg mL^{-1} in HFIP and Cy5-FF at 20.0 μg mL^{-1} in HFIP were injected in water to a final concentration of 2.0 mg mL^{-1} and 440.0 ng mL^{-1}, respectively.

For assembly dynamics, FF at 6.0 mg mL^{-1} was prepared by injecting FF at 100.0 mg mL^{-1} in HFIP in water in a well of a 8-well Labtek chamber. Once the FF fibers were formed (less than 5 minutes), Cy5-FF at a final concentration of 6.5 μg mL^{-1} or Cy3-FF at a final concentration of 0.65 μg mL^{-1} were added. After a short equilibration time (5 minutes) the supernatant was removed and FF in water at 0.7 mg mL^{-1} was added. Then half of the volume of Cy5-FF/FF fibers was mixed with half of the volume of Cy3-FF/FF fibers in another well of a 8-well Labtek chamber and images were taken at different time points. For controls, only Cy3-FF/FF fibers or Cy5-FF/FF fibers were imaged.

Epifluorescence microscopy. Epifluorescence images were acquired using a Nikon N-STORM system in Epi mode equipped with a Nikon 20x objective (air, 0.5 NA) and an epifluorescence lamp (Intensilight C-HGFIE). TexasRed filter was used to acquire images by stitching 18 x 18 = 324 frames with a

Hamamatsu ORCA-Flash 4.0 camera at 70 ms exposure time, resulting in a 6605 x 6605 pixels image (pixel size 0.8 μm).

TIRF microscopy. Fluorescent images were obtained using a Nikon N-STORM system configured for total internal reflection fluorescence (TIRF) imaging. Cy5-FF was illuminated by the 647 nm laser (nominal power, 160 mW) and Cy3-FF was illuminated by the 561 nm laser (nominal power, 80 mW). Fluorescence was collected by means of a Nikon 100x, 1.49NA oil immersion objective and passed through a quad-band pass dichroic filter (97335 Nikon). TIRF angle was adjusted to maximise the signal to noise ratio. Images were acquired onto a 256 x 256 pixels region (pixel size 0.16 μm) of a Hamamatsu ORCA- Flash 4.0 camera at 40 ms integration time.

Images of control Cy5-FF/FF fibers (with only Cy5) showed 15% crosstalk to the 561 channel. Thus, this 15% was subtracted in all the 561 channel images of self-assembly dynamics (Cy3-FF/Cy5-FF/FF fibers mixed in time). Concretely, the 15% image of the 647 channel was obtained and subtracted from the 561 channel image using ImageJ. Images of control Cy3-FF/FF fibers (with only Cy3) showed no crosstalk to the 647 channel.

PAINT microscopy. To perform PAINT imaging FF fibers were immobilized by adsorption onto the surface of a flow chamber assembled from a glass slide and a coverslip (24 mm x 24 mm, thickness 0.15 mm) separated by double-sided tape. PAINT images were acquired using a Nikon N-STORM system configured for total internal reflection fluorescence (TIRF) imaging. Cy5-FF was illuminated by the 647 nm laser (160 mW). Fluorescence was collected by means of a Nikon 100x, 1.49NA oil immersion objective and passed through a quad-band pass dichroic filter (97335 Nikon). Images were acquired onto a 256 x 256 pixel region (pixel size 0.16 μm) of a Hamamatsu ORCA- Flash 4.0 camera at 10 ms integration time. 60,000 frames were acquired for the 647 channel and PAINT images were analysed with the STORM module of the NIS element Nikon software. The NIS elements Nikon software generates a list of localizations by Gaussian fitting of blinking dyes in the acquired movie of conventional microscopic images. To avoid overcounting blinkings detected in consecutive frames are counted as single by the software.

Atomic Force Microscopy. 10 μL of the sample solution was dropped onto a freshly cleaved mica surface and adsorbed for a few seconds. The mica was then dried gently with nitrogen. A topographic image was recorded under a NanoWizard 3 BioScience AFM (JPK, Berlin, Germany) in the tapping mode at ambient temperature, with 512 × 512 pixel resolution and a scanning speed of 1.0 Hz.

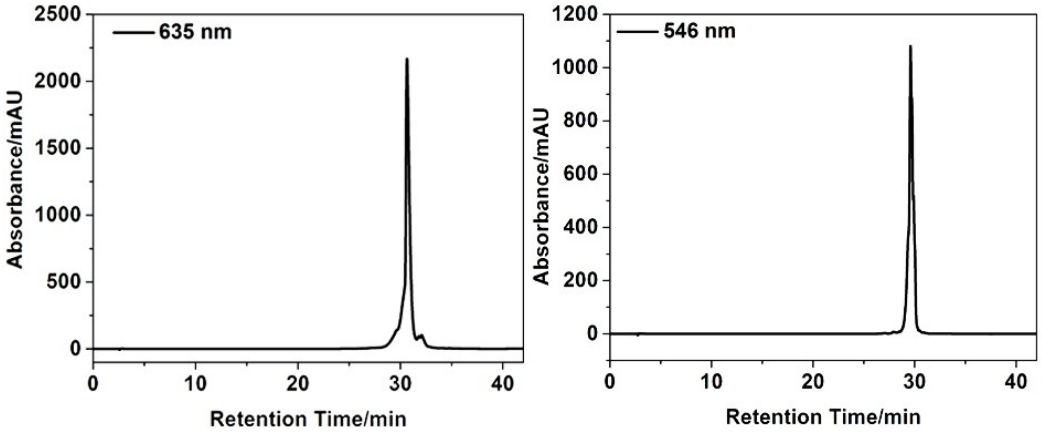

Figure S1. Reversed phase HPLC profiles of the synthesized Cy5-FF (left panel) and Cy3-FF (right panel). Note that a gradient elution mode was employed: eluent A, water, 0→3 min, 90% (A%), 3→33 min, 90%→10% (A%), 33→40 min, 10%→90% (A%); eluent B, MeOH. The monitoring wavelengths were set at 635 nm for Cy5-FF and 546 nm for Cy3-FF, and the flow rate was 1 mL min^{-1}. It is evident that the product peaks highly dominate the profiles and their relative areas are more than 95%, indicating the high purities of the synthesized compounds.

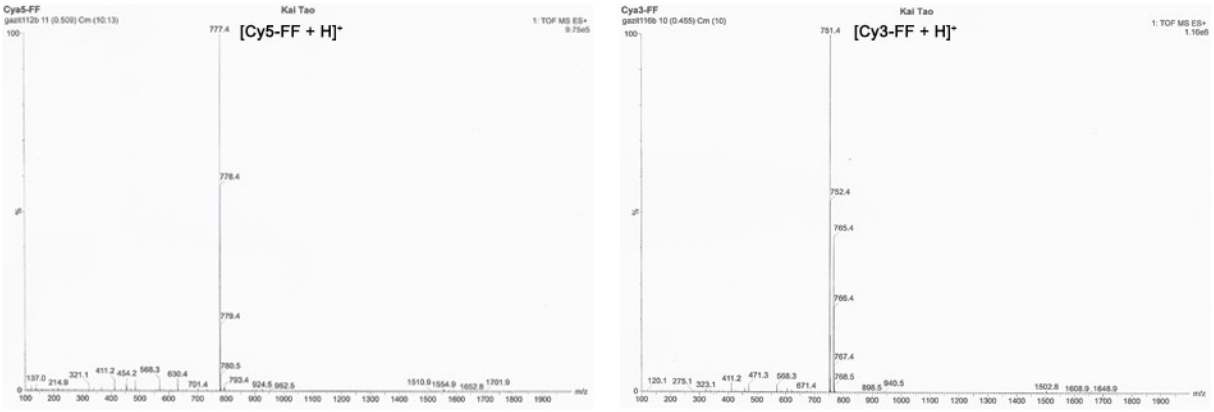

Figure S2. ESI-TOF spectra of the synthesized peptide probes. The observed molecular masses are consistent with the calculated ones, demonstrating the high purities of the synthesized compounds, well in accordance with the HPLC analysis described above:

Cy5-FF:

Expected masses $[M+H]^+$=777.44;

Observed masses $[M+H]^+$=777.4.

Cy3-FF:

Expected masses $[M+H]^+$=751.99;

Observed masses $[M+H]^+$=751.4.

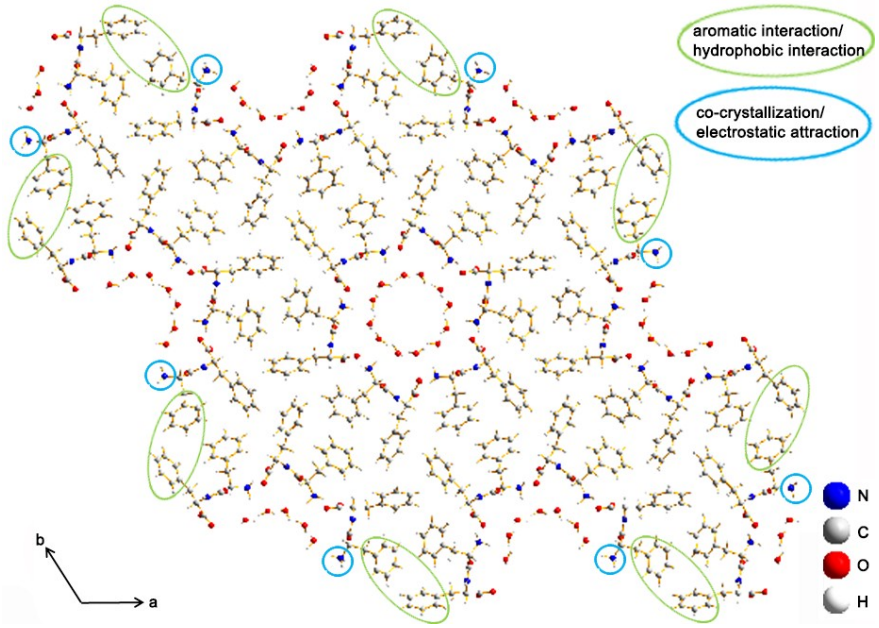

Figure S3. FF crystallographic structure showing the probable molecular mechanisms of Cy5/Cy3-FF anchoring at FF self-assemblies. Cy5/Cy3-FF can adsorb on the nanofiber surface through interacting with aromatic side-chains by aromatic interactions and hydrophobic interactions (labelled with green ovals); or through interacting with the N-terminal amino groups by electrostatic attractive interactions, or through co-crystallization by replacing the peripheral FF molecules (labelled with blue cycles). No matter what kind of mechanism, it can be concluded that the significant steric hindrance derived from the Cy5/Cy3 (see Fig. 1A in the main text) impedes the probe molecules to match the crystal structures, thus leading to the probes only distribute at the surface.

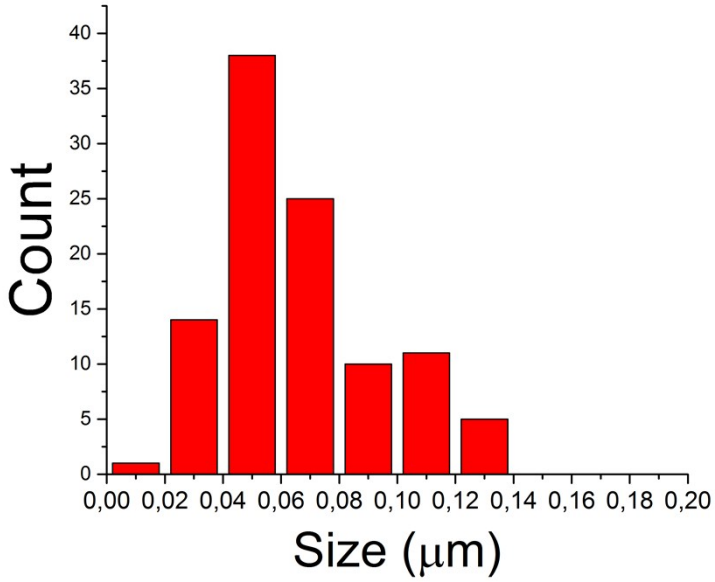

Figure S4. Histogram of the diameter size of peptide dots imaged by PAINT microscopy.

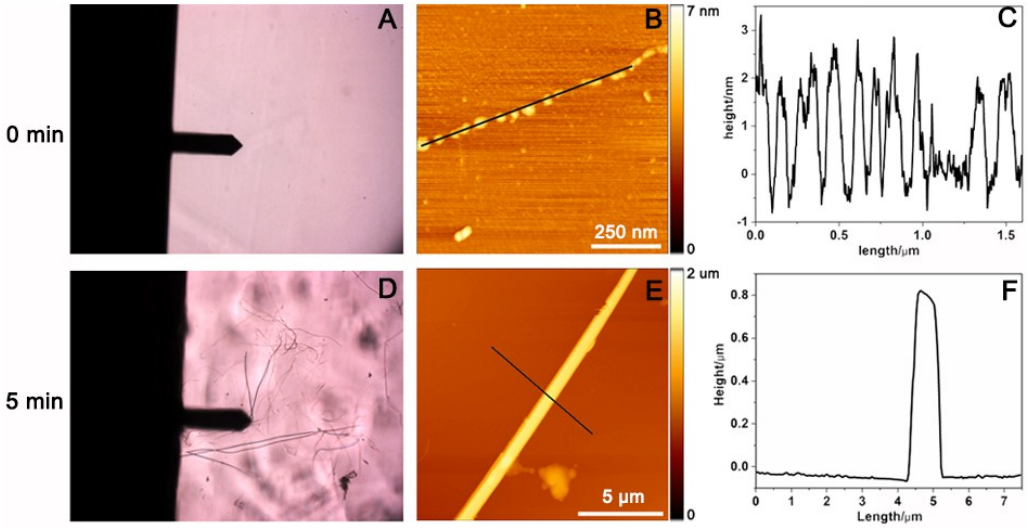

Figure S5. Time-resolved AFM characterization of FF self-assembly. Upper panel: immediately after diluting HFIP stock solution into water (0 min); Lower panel: 5 min after diluting HFIP stock solution into water. (A, D): optical microscopy images; (B, E): AFM images; (C, F) cross-sectional height profiles along the black lines in (B) and (E), respectively. It can be found that at the beginning after solvent switching, FF dipeptides formed small spherical dots, with few tens of nanometers of diameter; after 5 min, larger fibers could be found even from optical microscopy, showing the size of hundreds of nanometers.