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Supporting Information

Integrated Transmission Electron and Single-Molecule Fluorescence Microscopy Correlates Reactivity with Ultrastructure in a Single Catalyst Particle

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S1 Single Molecule Fluorescence movie

Movie of 200 frames played at 25 frames s^{-1}, in AVI format. The original output of the camera is a collection of TIF files. The movie was produced from the raw TIF files by the Localizer plugin of Igor Pro.

S2 Experimental details

1. Materials

Fresh fluid catalytic cracking (FCC) particles, containing zeolite ZSM-5 as the active phase, were supplied by Albemarle. Thiophene (99%) was purchased from Aldrich and used as received. Silicon nitride membranes were supplied by Silson Ltd. and had the following specifications: frame size 3×3 mm^2, membrane size 0.5×0.5 mm^2, frame thickness 200 µm, membrane thickness 50 nm.

2. Sample Preparation

The FCC catalyst samples were first calcined in a static oven at 823 K (ramp 1 K min^{-1}) for 48 h. Subsequently, the catalyst particles were embedded in Epofix, a two-component epoxy resin by mixing the resin and the hardener in a weight ratio of 25:3 and adding this mixture to <0.1 mg of catalyst powder at 294 K and atmospheric conditions in a small polyethylene vial. The samples were cured overnight at 333 K. Sectioning was performed using a Reichert-Jung Ultracut E microtome and a diamond knife (Diatome Ultra 35°, 4 mm size) under a 6º clearance angle with a cutting speed of 2 mm s^{-1}. Using a droplet of milliQ water, the sections were then placed on a silicon nitride (SiN) membrane. The SiN membrane containing the FCC particles was calcined in a static oven at 873 K (ramp 1 K min^{-1}) for 48 h to remove the epoxy resin, which is fluorescent, and any organic impurities. The addition of thiophene for the fluorogenic probe reaction was performed immediately prior to the experiment; 10 µL of pure thiophene was placed on top of the membrane containing the particles and allowed to dry for 5 min after which the SiN membrane was immediately placed in the vacuum chamber of the integrated setup. Both the staining reaction and the integrated SMF-TEM experiment were carried out at 294 K.

3. Methods

Prior to depositing the FCC thin sections, the SiN membranes were coated with a titanium oxide (TiO_2) layer using atomic layer deposition (ALD). Plasma-assisted ALD was carried out on a Flexal 2 (Oxford Instruments) with a separate load lock accessory. A cyclic deposition process was used with titanium isopropoxide and water as reactants, resulting in a 5 nm layer of TiO_2 on the complete surface of the SiN membrane (i.e. on the top and bottom).

Both transmission electron microscopy (TEM) and single molecule fluorescence (SMF) microscopy were carried out in a customized iCorr setup (FEI). This setup is modeled after the integrated setup used in the work by Karreman et al., however, instead of a confocal fluorescence microscope, a wide-field fluorescence microscope is now used to enable SMF microscopy capabilities.[1,2] A photograph of the setup is shown in Figure S1. The schematic in Figure S1 shows that the sample, held in a (standard) TEM holder, can be rotated 90º to enable both the TEM and SMF measurements.

Fluorescence microscopy images were recorded using an epifluorescence wide-field microscope with a 25× air objective (NA = 0.55). The sample was illuminated with a Cobolt 532 nm laser providing 3.5 kW cm^{-2} to the sample. The emitted fluorescence was passed through a dichroic mirror and an ET585/65 band pass filter (Chroma Technology Corporation) and recorded using a PCO 4.2 Edge (PCO-Tech) sCMOS camera. This field of view was cropped to 107×107 μm^2 (400×400 pixels) with a pixel size of 268×268 nm^2. The exposure time was experimentally determined to be optimal at 300 ms. TEM measurements were performed on a Tecnai 12 TEM (FEI) instrument. An 80 kV acceleration voltage was applied and images were taken using a 2K x 2K TEMCAM F214 (TVIPS).

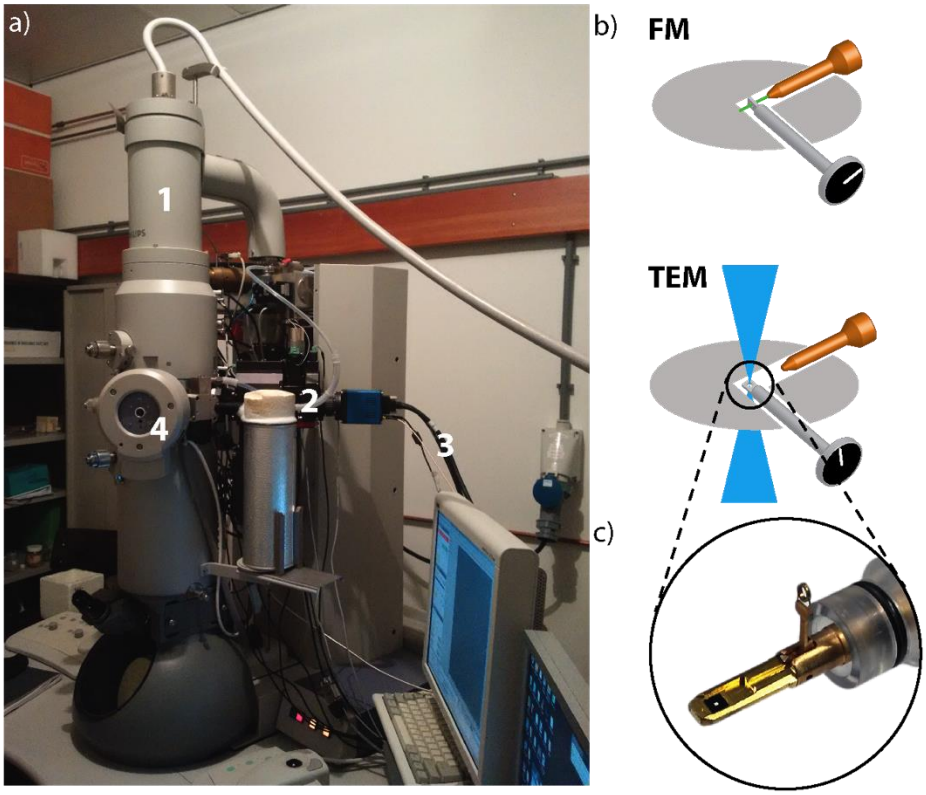

Figure S1. Overview of the integrated SMF-TEM setup that enables measuring integrated single molecule fluorescence (SMF) microscopy and transmission electron microscopy (TEM). a) A photograph of the setup shows the TEM column (1), the fluorescence detector (2), the fiber-optic cable through which laser light is transmitted (3) and the sample port (4). b) The sample can be rotated inside the setup to accommodate the two techniques. For fluorescence microscopy measurements, the sample is vertical; for TEM measurements, it is horizontal. c) A close-up of the standard TEM sample holder in which the silicon nitride membrane is placed.

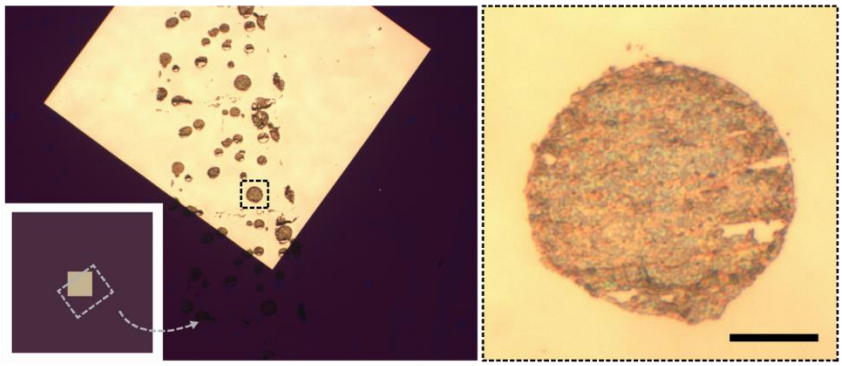

Figure S2. a) The silicon nitride (SiN) membrane used as sample support, after deposition of the thin section and subsequent calcination at 873 K. A silicon nitride membrane of 3×3 mm^2 was used, with a membrane of 0.5×0.5 mm^2 (see also Materials section). b) A zoom-in of the area marked in a, showing the FCC thin section that was used in the experiments. The scale bar is 20 µm.

4. Single Molecule Fluorescence Image Analysis

Figure S3 shows the histogram of the number of detected single molecule events over time, integrated over 400 frames. The average number of events per frame is approximately 10 in the beginning, after which there is a gradual decrease to 5-6 events per frame. This still significant number of events shows that over the course of the experiment, enough reactants are available for the fluorogenic probe reaction, even though the experiment is carried out in a vacuum chamber.

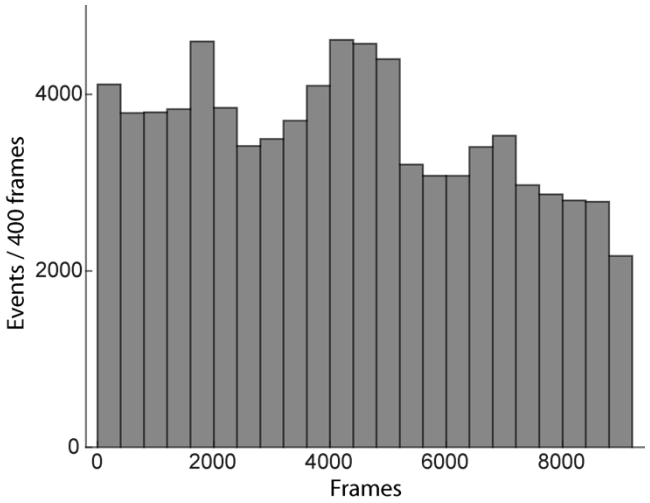

Figure S3. A histogram of the number of detected single molecule events over time. Each bar represents the sum of events detected in a subset of 400 frames.

Single molecule fluorescence microscopy analysis was carried out using the Localizer plugin of Igor Pro,[3] using the "SmoothSigma" segmentation algorithm and Gaussian fitting to localize single molecule events. Particles were found using 8-way adjacency; the standard deviation of the point spread function was 1 pixel. A "SmoothSigma" factor of 4.5 was experimentally determined to be optimal for detecting events, based on visual inspection of localized events in selected frames at different times in the movie. The localization error was calculated from the location of recurring events, and was estimated by Localizer to be 25 nm (Figure S4). Some small areas with continuously high fluorescence were detected by NASCA; these areas show no fluctuation in fluorescence, and are therefore most likely caused by a contamination rather than the thiophene oligomerization reaction. In contrast, SOFI does not show these areas, because they do not fluctuate. An example of this behavior can be observed in Figure 2d (main text), in the middle-left of the image. Drift correction was performed based on the localized events, using 82 sub-images with a pixel size 4× the original. This drift correction was then applied to each individual fluorescence microscopy image in order to apply this correction to both the average image and the SOFI analysis. A second order SOFI analysis was subsequently carried out at "maximum" quality; frames with saturated pixels (spikes) were removed from SOFI analysis.

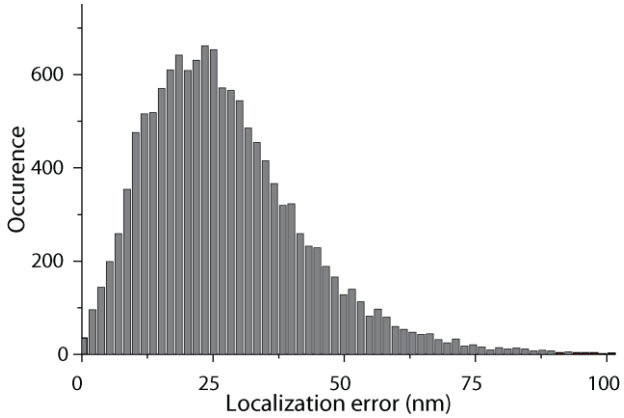

Figure S4. The localization error of detected single molecule events as calculated by the Localizer software, estimated by comparing localized emitters appearing in consecutive frames.

5. TEM structure classification

The classification of structural features observed in TEM was done manually. A region of interest within the catalyst particle thin section of 20 × 20 μm^2 was first divided into 1600 (40 × 40) segments of 0.5×0.5μm^2 each, with the size of these segments based on the size of the structural features observed as well as the feasibility of manual classification. Each segment was then classified as either zeolite material, matrix material or no material present. The classification was made solely based on structure, i.e. without taking into account the SOFI intensity of the areas. The SOFI intensity within each square was then summed and analyzed separately for each structure type.

6. Image overlay

Alignment of the results for both techniques was carried out using the cross-correlation algorithm “imregtform“ (multimodal method) in MATLAB and is shown in Figure S5. The overlay was then refined, based on structural features visible in both TEM and SMF: e.g., the area in Figure S5b. It must be noted here that in this type of correlated experiments, it is highly desirable to use fiducial markers (i.e. markers that are visible in both fluorescence microscopy and TEM) to fully automate the overlaying procedure and overlay with high accuracy. However, fiducial markers were not used in these experiments.

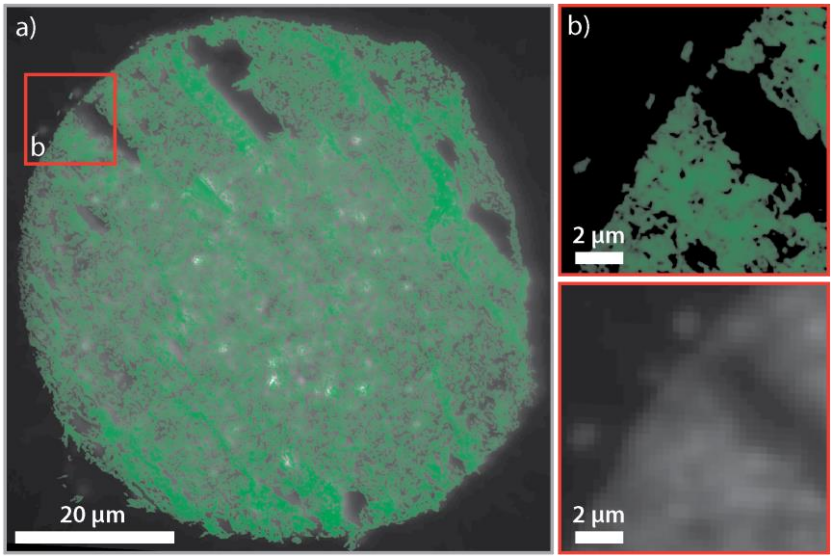

Figure S5. Details of the overlay of SMF and TEM. The TEM structure is shown in green for clarity; the average fluorescence is shown in gray scale. a) This image provides an overview of the overlay. b) This image shows an example of the visual marker at the edge of the thin section used to fine-tune the overlay.

S3 Additional Analysis

1. TEM masking using SOFI intensity

A thresholding procedure was used to segment the TEM images based on the corresponding SOFI intensity. Although the SOFI method is able to remove most of the background, because it is not fluctuating, low levels of background fluorescence remain. Therefore, the efficiency of removing this background by thresholding the TEM image at different intensity levels were assessed. Figure S6a shows the histogram of SOFI intensity with the chosen thresholds. The SOFI image above and below this threshold was then used as a mask for the TEM image. The left set of images (boxed red) in Figure S6b shows structural features corresponding to low reactivity, while the right set of images (boxed green) shows features corresponding to high reactivity in the thiophene oligomerization reaction. Structures identified as zeolite crystals are mostly visible in the right set of images. Upon increase of the threshold, the remaining areas in the image are seen to be ‘closing-in’ on these zeolite ultrastructures. Additionally, apparently unreactive zeolite ultrastructures can be identified in the left set of images (indicated with a blue arrow in Figure S6b). The thresholding approach shows the correlation between certain structural features and fluorescence intensity. The results are in agreement with the complementary approach detailed in the main text (Figure 3), in which the image is segmented based on the structural features instead of the SOFI intensity.

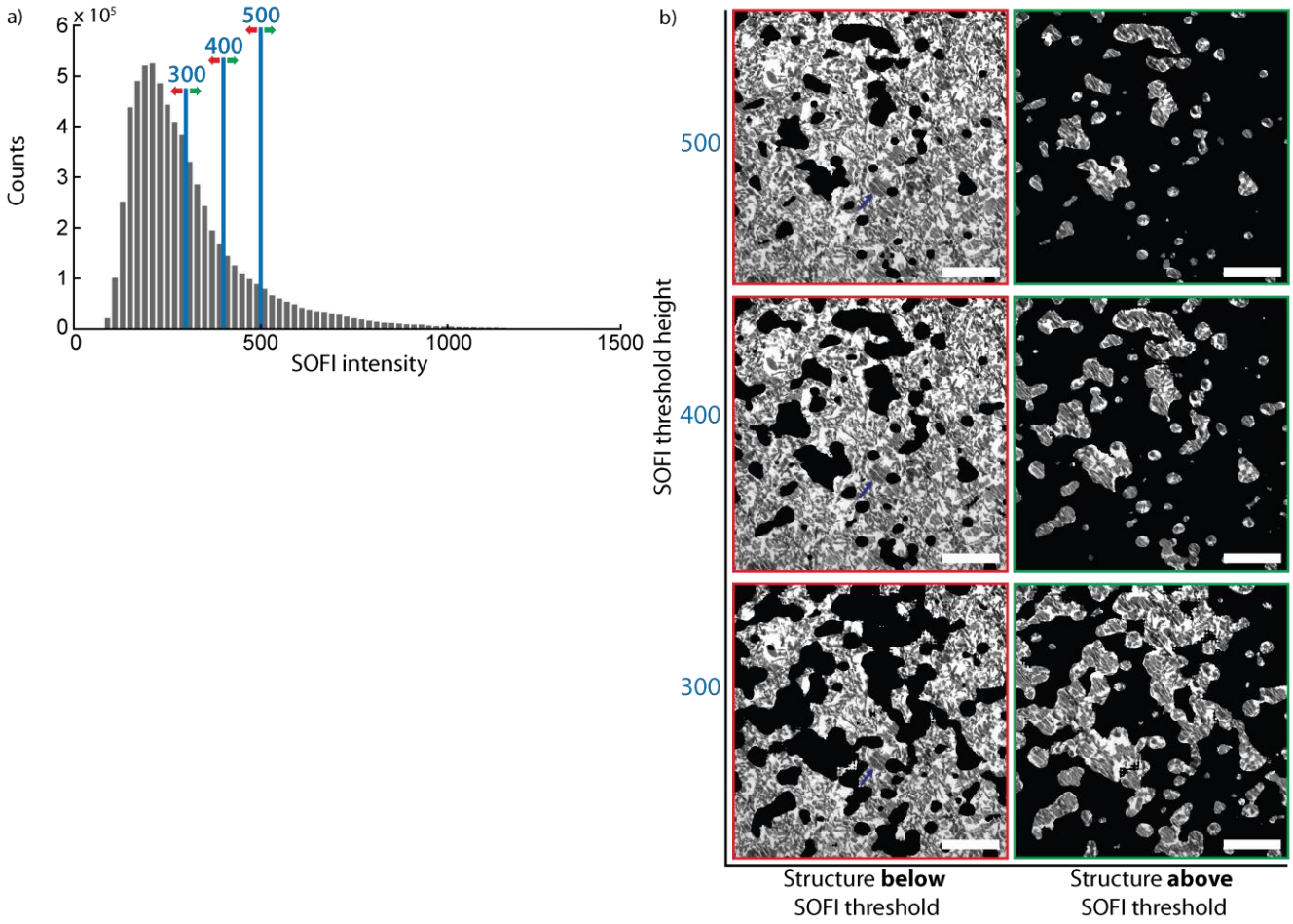

Figure S6. SOFI intensity can be used to identify structural features with high fluorescence activity. a) Histogram of the SOFI image intensities, with the thresholds defining high versus low intensity as indicated. b) Areas in TEM images with high (red, left side) and low (green, right side) SOFI intensity, for each threshold shown in a). The resulting images show structural features associated with either high or low reactivity. The scale bars are 5 µm.

S4. References

[1] M. A. Karreman, I. L. C. Buurmans, J. W. Geus, A. V. Agronskaia, J. Ruiz-Martínez, H. C. Gerritsen, B. M. Weckhuysen, *Angew. Chem. Int. Ed.* **2012**, *51*, 1428–1431.

[2] M. A. Karreman, I. L. C. Buurmans, A. V. Agronskaia, J. W. Geus, H. C. Gerritsen, B. M. Weckhuysen, *Chem. Eur. J.* **2013**, *19*, 3846–3859.

[3] P. Dedecker, S. Duwé, R. K. Neely, J. Zhang, *J. Biomed. Opt.* **2012**, *17*, 1–5.