Supplement

Supplementary Methods

1. Fluorescence labeling procedure
Fibrinogen, 5mg/mL in 0.1M NaCl, 1mM EDTA, 20mM triethanolamine-Cl, pH 7.5, was labeled at 4 °C with tetramethyl-rhodamine N-hydroxysuccinimide ester at molar ratios of 1:1, 2:1, 5:1, and 10:1. The labeling reactions were quenched after 1h by addition of tris(hydroxymethyl)aminomethane chloride to a final concentration of 80mM, then dialyzed against phosphate-buffered saline. Residual free dye was removed by desalting on columns of ToyoPearl HW40F (8 x 40 mm) in PBS. Fibrinogen was labeled with Alexa 488 according to the protocol for the Alexa Fluor-488 protein labeling kit from Molecular Probes. The bulk concentration of dye/fibrinogen was determined spectrophotometrically.

Stocks of fluorescent fibrinogen were prepared at bulk labeling ratios of 0.64, 1.18, 2.37 and 3.93 dyes/fibrinogen for Rhodamine labeled samples and at bulk labeling ratio of 0.3, 1.9, 2.2, 3.7 and 4.2 dyes/fibrinogen for Alexa labeled samples. All stocks of fluorescent fibrinogen had concentrations of approximately 1mg/ml and were stored at -80°C until used.

2. Preparation of platelet poor plasma (PPP) was done by collecting whole blood from several healthy donors into sodium citrate (final concentration 10 mM). We had at least 6 healthy donors (not taking for 3 months any drugs that interfere with clotting) recruited through posted signs and word of mouth. The donors were about equal female/male, ages 21-65, and all signed consent forms. The blood donation protocol and consent form were approved by a University of Pennsylvania Institutional Review Board. Immediately after collection, blood was spun at room temperature for 15 min at 160g and then the supernatant was spun down at 10,000g for 10 min and pooled. The concentration of fibrinogen in our PPP was 2.5mg/ml and was kept frozen at -80°C until used.

3. Molecular calibration
The average value of total intensity of a bleaching step, $I_{\text{bleaching step}}$, and the distribution of the bleaching steps obtained experimentally were used to develop the molecular calibration. A binomial distribution was used to calculate the probability of labeling of one site of fibrinogen. Assuming that $n$ is the number of equally reactive available sites for labeling and $q$ is the probability of labeling one site, the probability of labeling no sites, one site, two sites, etc. of the fibrinogen molecule will be, respectively: $C_n^0(1-q)^n$, $C_n^1q(1-q)^{n-1}$, $C_n^2q^2(1-q)^{n-2}$, etc. where $C_n^k$ are the Newton binomial coefficients, $k$ is the number of labeled sites and $\sum_{n,k=0}^n C_n^k q^k (1-q)^{n-k} = 1$.

Assuming that labeling is proportional to observations, the ratio of single- to double-labeled fibrinogen molecules, $C_n^1q(1-q)^{n-1}/C_n^2q^2(1-q)^{n-2} = 2(1-q)/q(n-1)$, can be correlated with single-molecule experimental data (1-step bleaching events) / (2-step bleaching events), which will give, for a particular $n$, the probability of actively labeling...
of one site, \(q\), and further the probability of actively labeling a fibrinogen molecule with \(k\) dyes, \(p_k\).

Summing for all molecules present in a fiber, each with its probability of labeling, \(p_k\), we obtain the intensity \(I\) of one fibrin fluorescent fiber to be

\[
I = I_{\text{bleaching step}} M \alpha \sum_{k=0}^{n} k p_k
\]

where \(I_{\text{bleaching step}}\) is the intensity of the bleaching step (corresponding to one dye attached to the fibrinogen molecule), \(M\) is the number of molecules in the fiber under observation, \(n\) is the number of the active sites, \(p_k\) is the probability of labeling a fibrinogen molecule at \(k\) sites: \(p_k = C_n^k q^k (1 - q)^{n-k}\) and \(\alpha\) is a factor that takes into consideration the unlabeled fibrinogen from plasma, since we are mixing the labeled fibrinogen with plasma in our experiments. \(\alpha = M_f / M\), where \(M_f\) is the total number of molecules within the fluorescently labeled part of the sample. \(\alpha = 1\) for a sample without plasma and <1 for a sample that has plasma added. In our case \(\alpha = 0.038\). By using this analysis, the molecules that are present but not fluorescent, according to the distribution of labeling, within the fluorescent sample and also unlabeled fibrinogen from plasma are introduced in the calibration term and contribute to give the total number of molecules within the fiber, \(M = I / (I_{\text{bleaching step}} \alpha \sum_{k=0}^{n} k p_k)\).

An analysis of the values that can be taken by \(n\) in the particular case of fibrinogen and the impact on probability calculations of labeling is presented in Supplementary Data in Fig S3.

4. Calculation of the number of molecules in cross-section of fibers

The number of molecules in cross-section of a fiber, \(c\), is also the number of parallel rows of molecules constituents of protofibrils that form the fiber:

\[
c = M / \text{number of molecules in a row} = M / (L / l_{\text{fibrin}}) = M l_{\text{fibrin}} / L
\]

where \(L\) is the length of the fiber analyzed and \(l_{\text{fibrin}} = 45\) nm (1).

5. Calculation of fibrin fibers diameters

We started by calculating the fibrin molecule diameter first. For this, we calculated the volume of one molecule as the molar volume divided by the No. Avogadro (NA) and the molar volume was calculated from the molecular weight (MW) and density (partial specific volume) (\(\rho\)):

\[
V_{\text{molecule}} = V_{\text{molar}} / \text{NA} = \text{MW} \rho / \text{NA} = [340000g/mole 0.72cm^3/g] / [6.02 \times 10^{23} \text{molecules/mol}]
\]

\[
= 4.1 \times 10^{-19} \text{cm}^3 = 410\text{nm}^3.
\]

Assuming the molecule geometry is cylindrical we have:

\[
V_{\text{molecule}} = l_{\text{fibrin}} \pi r^2
\]

which will give a molecule radius \(r = 1.7\) nm and a diameter of \(d = 3.4\) nm. We used \(l_{\text{fibrin}}\) MW and \(\rho\) values according to Weisel\(^2\).

To calculate the diameters of fibers, we assumed that the fiber is uniform with the protein density determined experimentally\(^3,4\) and the cross section of the fiber is circular. With these we have \(\pi D_p^2/4 = c \pi d^2/4\) where \(D_p\) is the average diameter of the “protein packed” fiber that has no water content, which gives \(D_p = dc^{1/2}\). At this point we have to take into account that fibrin fibers are highly hydrated structures. Whereas turbidity studies
estimate that fibrin network contains 70-80% water\(^4\) (percentage related to volume, not to crosssectional area of fiber), electron microscopy studies suggest that fiber water content is two thirds only\(^5\). Using an average value of 70% for water content, we have \(V_{\text{hydrated fiber}} = (10/3)V_{\text{protein}}\) that is \(L\pi D^2/4 = (10/3)L\pi D_p^2/4\) which will give a diameter for the hydrated fiber of \(D = 1.8 D_p\).

In a theoretical model based on crystallographic data, Yang et al.\(^6\) estimate the dimensions between protofibrils within fibrin fibers. By using their model in our calculations, our fibers would be 12% thinner then what we calculated above (with water included), which is in reasonable agreement, since experimental data coming from spectrometry and small angle x-ray scattering experiments suggest that the real distances between protofibrils are actually bigger then what is obtained from crystallographic data, and also variable due to conditions\(^5\).

6. **Considerations on imaging in TIRF – from single molecule to fibers**

A basic question in single molecule fluorescence studies is that, if several-step bleaching happen in the same area, how often could that be due to several molecules that fell in the same area instead of one molecule with multiple labels.

To address this, ultracentrifugation was used to sediment the aggregates within the samples and leave single molecules in the supernatant. The small amount of molecules that are introduced in the TIRFM chamber minimizes the chance of one bright spot in the image to be the result of two or more molecules that fall in the same place. This issue was addressed experimentally with molecules fluorescently labeled specifically, that allow a specific number of dyes to be attached. Single myosin molecules, which can be labeled specifically at both heads with one dye each, thus each molecule being double labeled, present in statistics less than 1% of three or more-step bleaching\(^7\), indicating that the number of molecules that fall in the same place is insignificant. In our case, in the experimental distributions obtained for fluorescent fibrinogen, even at larger bulk labeling ratios, the number of multiple-step events is much smaller than the one-step and two-step bleaching events and therefore less significant. Also, analysis for calibration is done on one-step bleaching and two-step bleaching events, that are predominant, so even though there is a small chance that some multiple-step events come from several molecules that fall in the same place, they are not directly used in our calculations.

The fibers we observe in TIRFM have variable diameters and some, in the diffusive micromixing regime, grow up to 900 nm in diameter or more. To estimate how accurately the big fibers are seen in the TIRFM evanescent field we measured their dimensions by atomic force microscopy imaging - both in physiological buffer and glutaraldehyde fixed and it showed that all fibers on the surface, including fibers as thick as 1 µm diameter have heights under 80-90 nm\(^8\), proving without doubt that they are in the TIRFM range of observation. Fibrin fibers are probably flattened because the protofibrils comprising them are very loosely packed (due to the large water content of fibers) and hence the fibers become flattened due to their own weight even in liquid.

**Supplementary Data**

In order to eliminate any possible dye artifacts, we performed parallel experiments by using two dyes, tetramethyl-rhodamine and Alexa-488. We obtained similar results with both dyes, as presented. Figure S1 presents the bleaching rate and average intensities of
one step bleaching events for both dyes. The time constant for each dye was determined by fitting with an exponential the decay curve obtained by summing up the signals obtained from over 40 one-step bleaching events (Fig S1 A) and was used for bleaching correction of the raw data, the original images of fibers. Average values of intensities of both dyes (Fig S1 B) are intensities of more then 40 one-step bleaching events, in each case.

Bleaching event distributions for several bulk labeling ratios of Alexa-488-labeled fibrinogen showed the same trend as for the Rhodamine-labeled samples (Fig S2). The one-step bleaching events are predominant for all the bulk labeling ratios used. When we tried a larger bulk dye/fibrinogen ratio, we obtained a large amount of aggregation in the samples, so under our labeling conditions, functional labeling was limited to 4 dyes/molecule.

The probability of labeling of fibrinogen molecules can be calculated for different numbers of available binding sites on the fibrinogen molecule. Fibrinogen has a maximum of 212 available sites for nonspecific labeling (amino groups); however not all of them are equally exposed on the surface of the molecule, or equally reactive. We used n=4 sites for our calculations since the maximum bulk labeling ratio used was 4 dyes/fibrinogen and the predominant labeling turned out to be lower, with one dye per molecule according to our single-molecule analysis of the bleaching events; however calculations with n=212 sites gave similar results. For each population we can estimate the amount of molecules not labeled, the ones that have one label, two labels, etc. For the 0.3 bulk labeling ratio with Alexa 488, most of the fibrinogen molecules were not labeled (as expected), followed by molecules labeled at only 1 site (Fig S3).

**Supplementary Figures**

Fig. S1. *Characterization of bleaching of Alexa-488 and Rhodamine dyes.* Bleaching rate (A) and total intensity values for one step bleaching events (B) for Alexa-488 and Rhodamine dyes attached to fibrinogen molecules under our TIRFM illumination.
conditions. Alexa 488 dye is more intense in TIRFM but bleaches much faster than Rhodamine. Error bars in graph (B) are standard deviations.

**Fig. S2.** Distributions of bleaching events for Alexa-488 labeled fibrinogen. Bleaching event distributions for several bulk labeling ratios of Alexa-488-labeled fibrinogen showed the same trend as for the Rhodamine-labeled samples.

**Fig. S3.** Probability of labeling of the fibrinogen molecules for different number of binding sites available on the molecule. For the bulk labeling ratio of 0.3 dyes/molecule using Alexa-488, most of the fibrinogen molecules were not labeled, as expected,
followed by molecules labeled at only 1 site, for both cases, i.e. 4 sites or 212 sites available for binding. No significant difference in labeling was obtained for assuming either 4 or 212 sites of labeling.

References