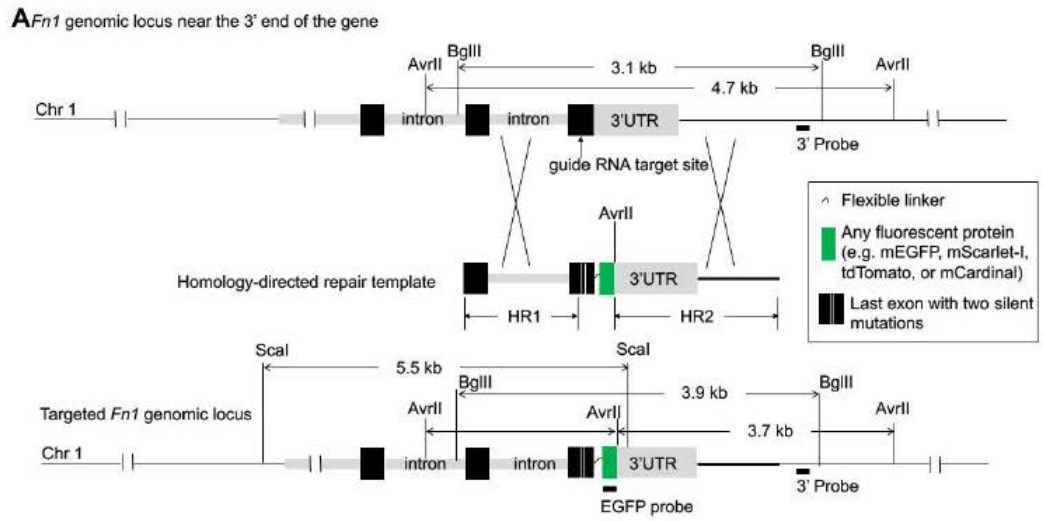
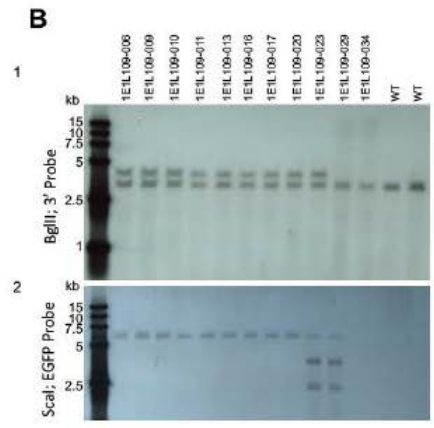
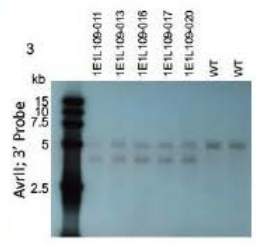
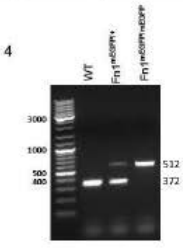
Supplemental Figure 1





Southern blot strategy			
Restriction enzyme	Probe	WT	Targeted
ScaI	EGFP Probe(5')	--	5.5kb
AvrII	3'Probe	4.7kb	3.7kb
BglII	3'Probe	3.1kb	3.9kb





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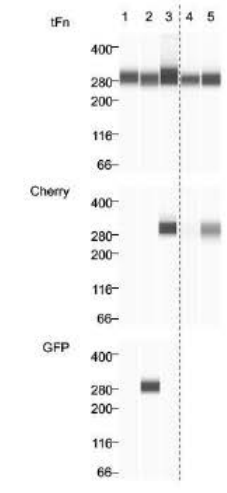
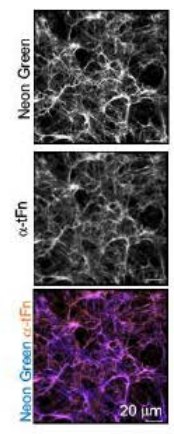
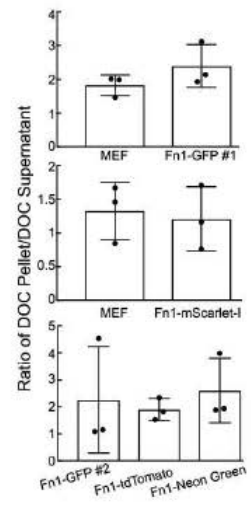
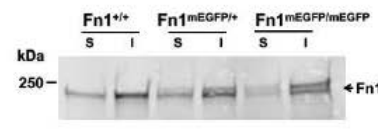
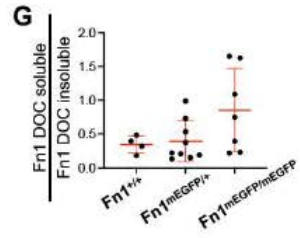
Fn1mEGFP/mEGFP x Fn1mEGFP/+ n=22	
Fn1mEGFP/+	Fn1mEGFP/mEGFP
12	10











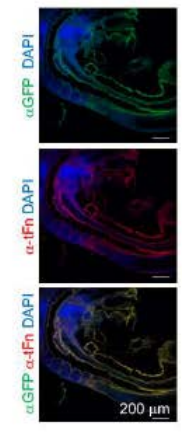


Fig. S1. Construction of Fn1^{mEGFP} allele. A. Targeting construct. **B1-B3**. Southern blots. **B1.** Southern blot with 3' probe after digestion with Bgl II. **B2.** Southern blot with GFP probe after digestion with Sca I. **B3.** Southern blot with 3' probe after digestion with AvrII. **B4.** Diagnostic PCR detecting Fn1$^{+/+}$; Fn1$^{mEGFP/+}$; and *Fn1$^{mEGFP/mEGFP}$*. **B5.** *Fn1$^{mEGFP/mEGFP}$* mice are obtained at a Mendelian ratio in the cross between *Fn1$^{mEGFP/mEGFP}$* and *Fn1$^{mEGFP/+}$* mice. **C-H. Fn1-FP fusion proteins behave like wild-type Fn1 in biochemical assays and *in vivo*.** CRISPR was used to knockin mEGFP, tdTomato, mNeonGreen, or mScarlet-I at the 3' of Fn1, replacing the termination codon in MEFs (see Sup. Fig. 1). **C.** Western blot of cell extracts: 1) wild-type MEFs, 2) Fn1^{mEGFP} MEFs, 3) Fn1^{tdTomato} MEFs, 4) wild-type MEFs, 5) Fn1$^{mScarlet-I}$ MEFs. **D.** Fn1^{mNeonGreen} MEFs were stained with antibody to total Fn1; Native mNeonGreen fluorescence of Fn1-mNeonGreen proteins co-localizes with Abcam monoclonal antibody to tFn1. **E.** Fn1-FP fusion proteins behave like wild-type in DOC matrix assembly assays. **F-G**. Fn1-mEGFP proteins extracted from E9.5 embryos behave like wild-type in DOC matrix assembly assay. **F.** E9.5 embryos were solubilized using DOC lysis buffer and DOC-soluble and insoluble fractions were resolved on a 4-12% SDS-PAGE gel followed by a Western blot using Abcam monoclonal anti-Fn1 antibody. **G**. Quantification of the fraction of Fn1 DOC-soluble Fn1 to DOC-insoluble Fn1. Each dot is an embryo. One-way ANOVA analysis showed no statistical differences between samples from different genotypes. **H.** E9.5 Fn1$^{mEGFP/+}$ embryos were stained with Abcam monoclonal antibody to Fn1 and anti-GFP antibody to detect Fn1 and GFP proteins; Fn1-mEGFP and Fn1 co-localize and are expressed in the expected pattern.

Supplemental Figure 2

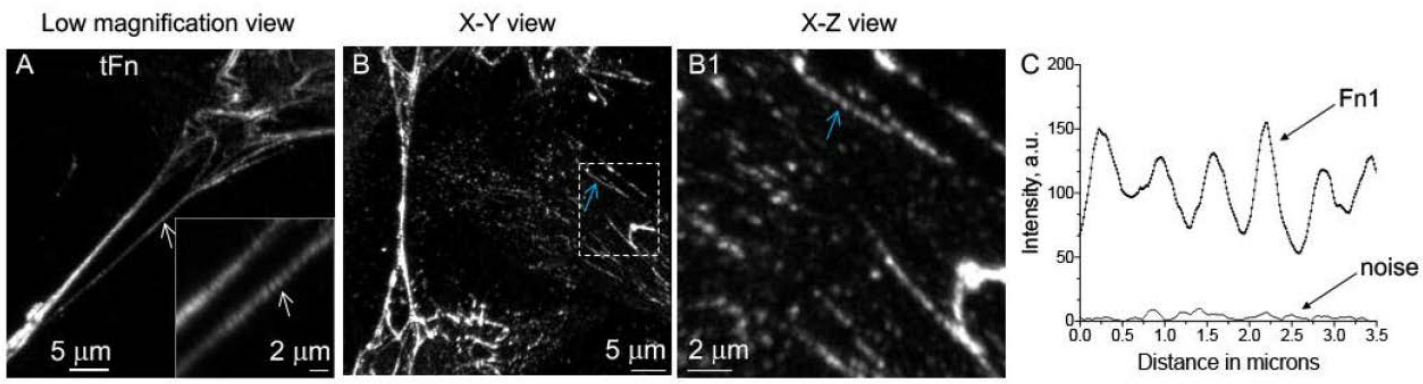
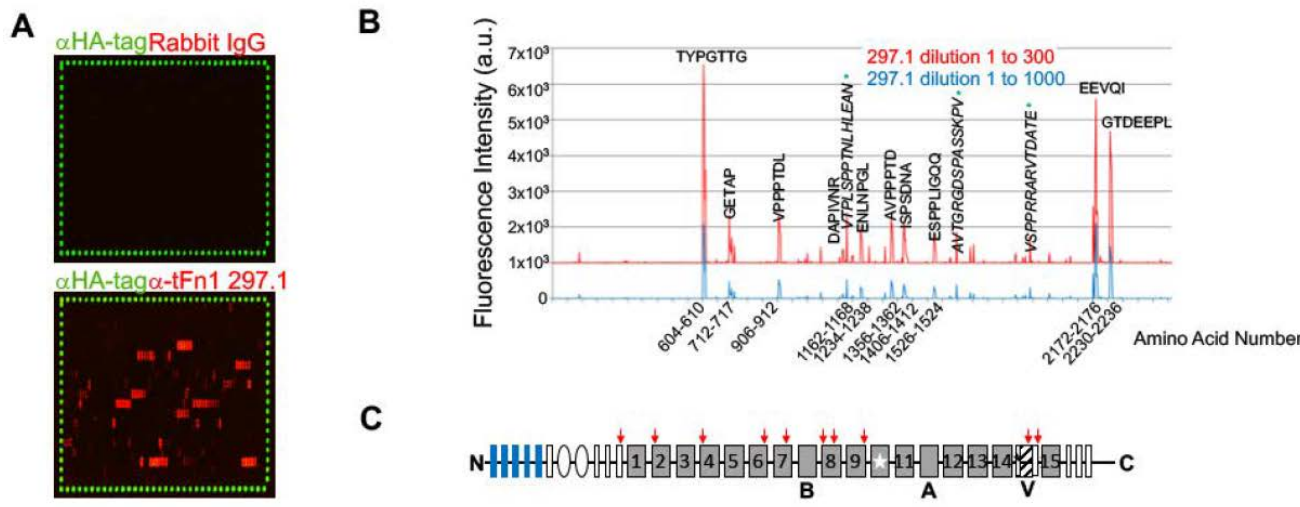
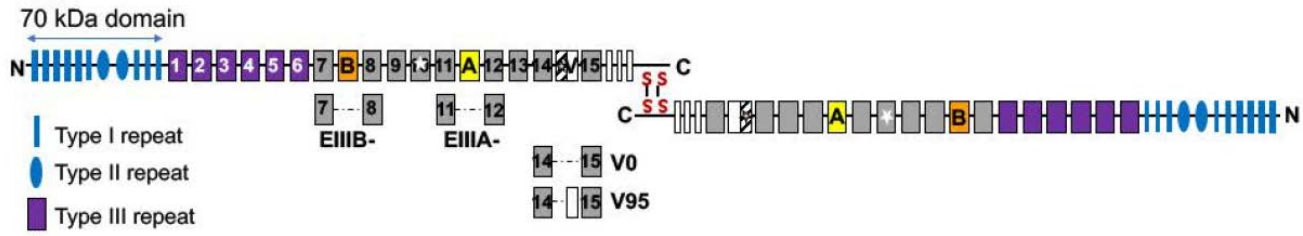


Fig. S2. Beaded architecture of Fn1 visualized by Zeiss Airyscan. Wild-type MEFs were plated for 48 hours on gelatin-coated glass, fixed and stained to detect Fn1 using the Abcam monoclonal anti-Fn1 antibody, and imaged with the Airyscan modality on Zeiss confocal microscope. **A.** Arrow points to a Fn1 fibril between cells, magnified in the inset. Note the beaded appearance of this fibril, arrows in **A**. Box in **B** is expanded in **B1** and rotated to show the x-z axis, blue arrow points to a beaded fibrillar adhesion. **C.** Intensity profile of the Fn1 fibril marked by the blue arrows in **B** and **B1**. Panel **C** shows that Fn1 fibrils consist of regions of high and low fluorescence intensity.

Supplemental Figure 3





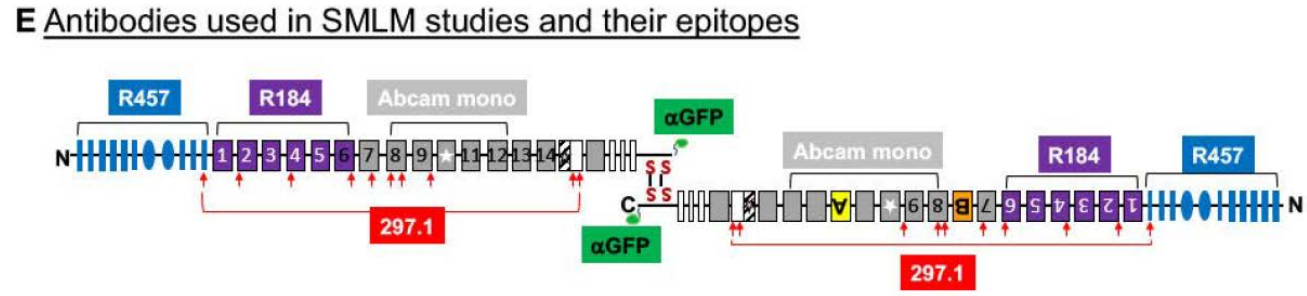


Fig. S3. 297.1 antibody recognizes multiple epitopes along the Fn1 molecule. A. Custom-prepared peptide arrays fabricated to contained 15-amino acid peptides with 13-amino acid peptide-peptide overlap, spanning the entire mouse Fn1 sequence. HA-tag peptides were spotted around the perimeter for control purposes. Arrays were incubated with anti-HA-tag antibodies followed by the appropriate secondary antibody (green) and with either rabbit IgG (top panel) or 297.1 antibodies (bottom panel) and the appropriate secondaries, red. **B.** Multiple strong to very strong antibody responses against epitope-like spot patterns formed by adjacent peptides with the consensus motifs annotated in the intensity plot at high signal-to-noise ratios; We also observed a few atypical interactions with peptide as highlighted in *italics and marked by asterisks*. Amino acid positions of epitopes are marked on the x-axis. **C**. Positions of 297.1 epitope-binding sites are mapped on mouse Fn1 molecule. The splicing of the V region is depicted according to the mouse mRNA tracks in GENCODE VM27. Schematic representations of Fn1 dimers and antibody binding sites. A. Fn1 is secreted as a dimer. Two disulfide bonds at the C-terminus hold Fn1 molecules in an anti-parallel orientation. 70 kDA Fn1 assembly domain is marked in blue and by a double-headed arrow. Fn1 type III repeats are numbered. Splice variants characterized in mice are shown. **D.** Schematic of binding sites for the antibodies used in SMLM studies. All antibodies except one are polyclonal, see Table S4. Arrows mark mapped epitopes. Brackets mark to GFP. regions of Fn1 recognized by the following antibodies: R457 is a rabbit polyclonal antibody raised to the 70 kDA domain of Fn1; R184 is a rabbit polyclonal raised against the first six type III repeats of Fn1 (purple); 297.1 is a rabbit polyclonal antibody raised to the full-length rat plasma Fn1; The binding sites of 297.1 (red arrows) were mapped in this study; Abcam mono is a monoclonal antibody recognizing an epitope within the marked region. aGFP is a polyclonal antibody recognizing EGFP.

Supplemental Figure 4

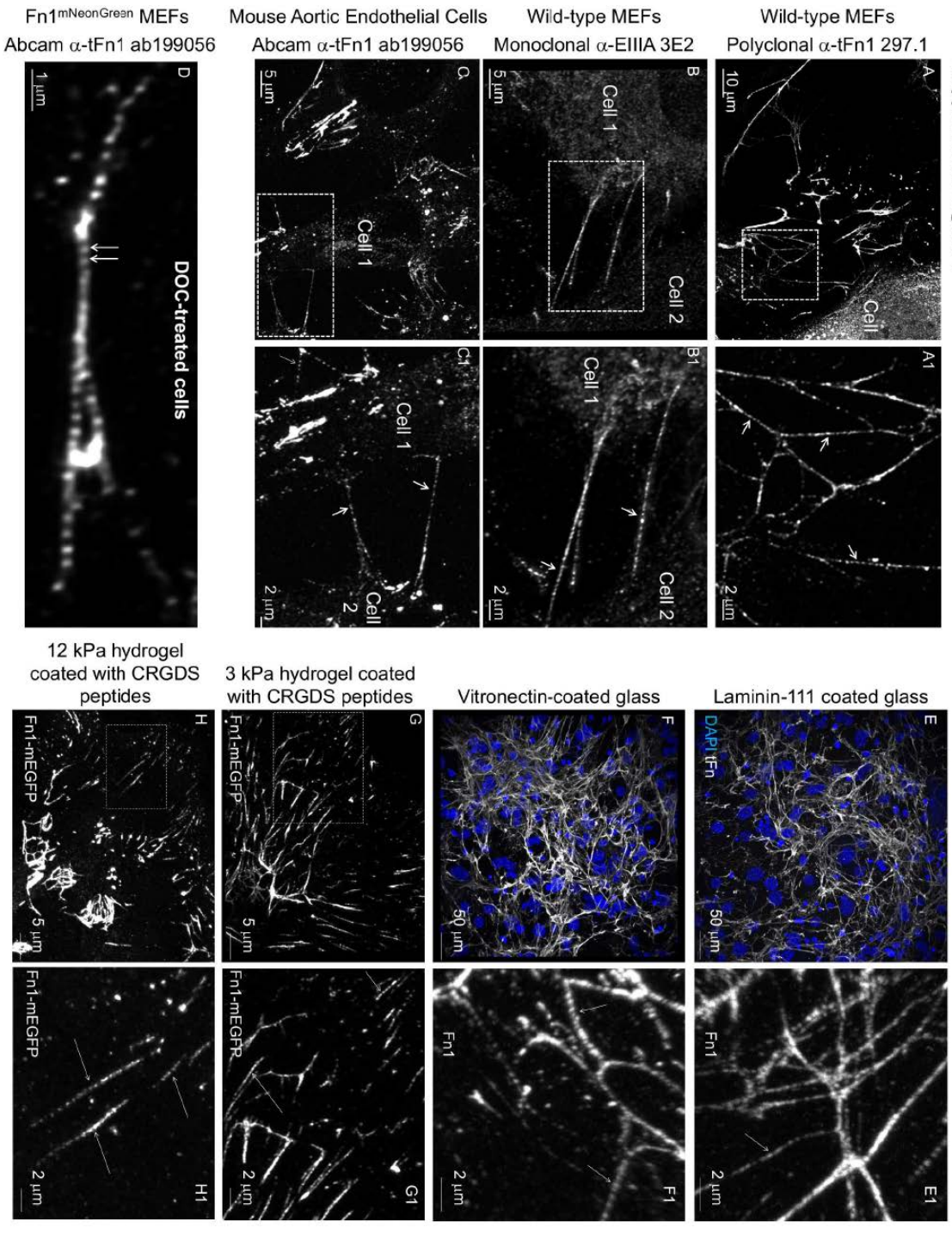
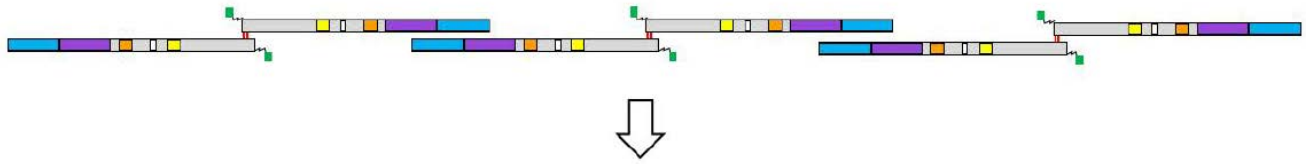


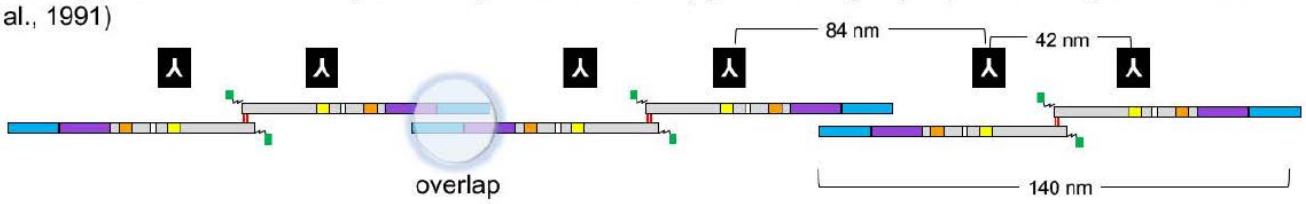
Fig. S4. The beaded architecture of Fn1 fibrils is seen with multiple antibodies in different cell types and is retained in the absence of cell contact. Cells were plated on glass without coating. **A** –**B.** Fn1 secreted by wild-type MEFs and deposited **A)** on glass or **B)** located between two cells. **C.** Fn1 fibrils between endothelial cells. Boxes in A-C are magnified in **A1-C1**. Arrows point to fibrils deposited in the intercellular space (**A1-C1**). **D.** Fn1 fibril imaged following the treatment with 2% deoxycholate for 10 minutes and staining with Abcam monoclonal antibody to Fn1. Note the beaded architecture of the long Fn1 fibril (arrows). Solubilization of cell components by this treatment is shown in **Movie 3**. All images were collected using 100x oil objective, NA 1.49, pinhole size 0.8 Airy units, and sampling resolution of 40 nm/pixel in x,y. **E-H. The beaded architecture of Fn1 fibrils is observed when cells are plated on different substrata**. **E-E1**. Wild-type MEFs plated on laminin-111 for 48 hrs. **F-F1.** Wild-type MEFs plated on vitronectin for 48 hrs. Cells in **E-E1, and F-F1** were stained using Abcam monoclonal anti-Fn1 antibody. **E-F.** Low magnification images were recorded with 40x oil objective, **E1-F1.** High magnification images were captured using 100x oil objective, NA 1.49; pinhole was set to 0.8 Airy units, and sampling was done at 40 nm/pixel in x,y (See Methods). **G-H.** *Fn1*$^{mEGFP/+}$ MEFs were plated for 16 hours on hydrogels derivatized with GRGDSPC peptides (See Methods). Native mEGFP fluorescence was imaged using 100x oil objective, NA 1.49, pinhole size 0.8, and sampling at 40 nm/pixel in x,y). **G** – **G1.** 3 kPa hydrogel; **H** – **H1** 12 kPa hydrogel. Arrows point to examples of beaded fibrils.

Sup. Figure 5

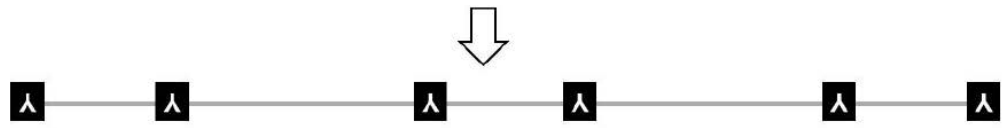
A Model proposed by Dzamba et al., 1991: Fibrils form by extended Fn1 dimers aligned in a periodical manner with regions containing N- termini alternating with regions containing C- termini. Green tail at the C-terminus represents mEGFP



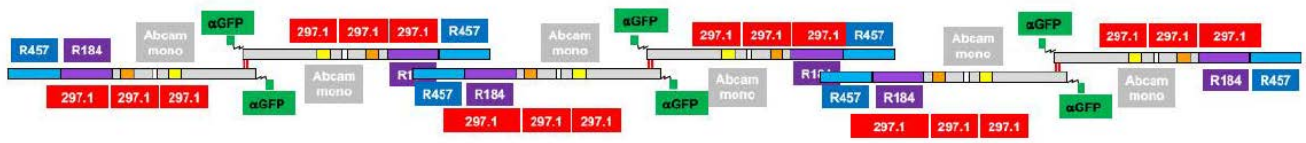
B Dzamba et al., 1991 model posits that periodical alignment of extended Fn1 dimers results in periodical staining pattern of Fn1 fibrils by domain-specific antibodies (Y) in EM images (adopted from Fig. 4A in Dzamba et al., 1991)



Dotted appearance of Fn1 fibrils seen by immunoelectron microscopy is due to the periodical distribution of region-specific epitopes along the extended Fn1 molecules in a fibril (inverted Y represents a bound antibody)



C Dzamba et al., 1991 model predicts that labeling of Fn1 fibrils by the five antibodies depicted below would produce uniformly-labeled fibrils



If all of the antibodies in the 5-antibody cocktails are detected with the same color, a fairly-uniform labeling of Fn1 fibrils would be expected according to the model of Dzamba et al., 1991 and Fruh et al., 2015

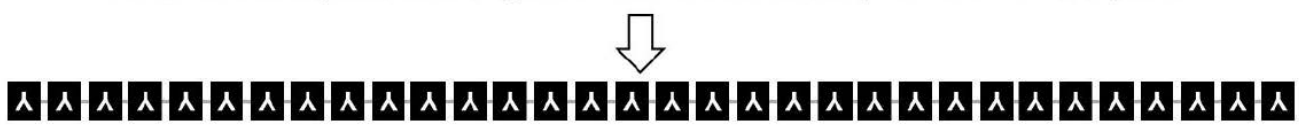
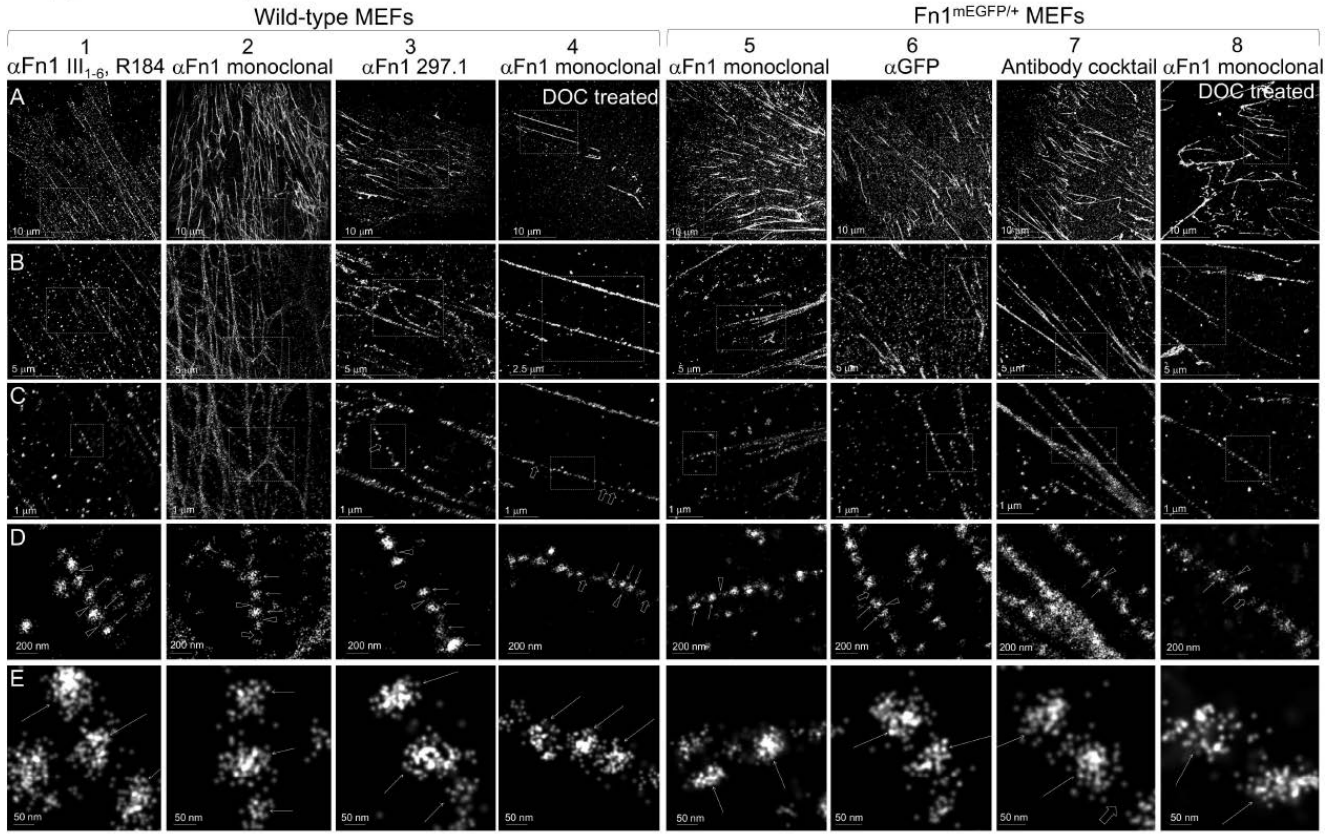


Fig. S5. Illustrations of fibronectin assembly model proposed by Dzamba et al., 1991.

A. Fn1 fibrils were proposed to arise from aligned and extended Fn1 molecules. According to this model, Fn1 fibrils look like ropes made of LEGO pieces, in which each Fn1 protein domain is a LEGO piece of a different color. **B.** Localizations of anti-EIIIA antibody binding epitopes along Fn1 fibrils according to the model in **A. C.** The use of multiple antibodies is predicted to result in a uniform non-periodical staining if the model shown in **A** were correct.

Supplemental Figure 6



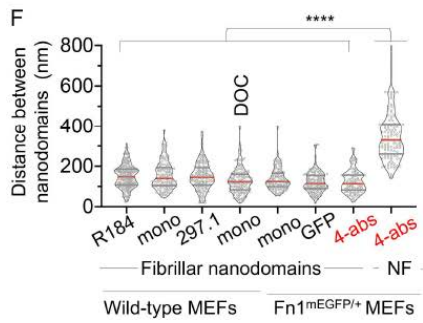


Fig. S6. The periodical architecture of Fn1 fibrils in see in wild-type cells and in fibrils devoid of cells. Wild-type or *Fn1*$^{mEGFP/+}$ MEFs were plated on glass for 16 hrs, fixed, and stained with different antibodies to Fn1 followed by Alexa 647-conjugated secondary antibodies. Columns 4 and 8 show cells treated with 2% DOC prior to fixation. Cells were imaged using SMLM imaging protocol II**. A.** zoom-out views to show the overall appearance of Fn1 fibrils. **B-E.** Successive magnifications of fibrils are shown in row (**A**). Arrows in **D** point to nanodomains magnified in **E;** arrowheads in **D-E** point to Fn1 localizations between nanodomains, wide open arrows point to Fn1-free zones between nanodomains in a fibril. **F.** distances between nanodomains within fibrils or non-fibrillar (NF) nanodomains, **** $p<10^{-4}$, Kruskal-Wallis test, with Dunn's correction for multiple testing. Please note that nanodomain sizes appear larger in these images than in Fig. 3 – 6 because the resolution was lower due to the use of high laser power during bleaching and imaging steps as described in Material and Methods SMLM protocol II; FRC-measured resolution in these images ranged between 40-50 nm. Antibody cocktail contained: R184 (1:50), Abcam mono (1:300), 297.1 (1:100) and anti-GFP (1:300) antibodies.

Supplemental Figure 7

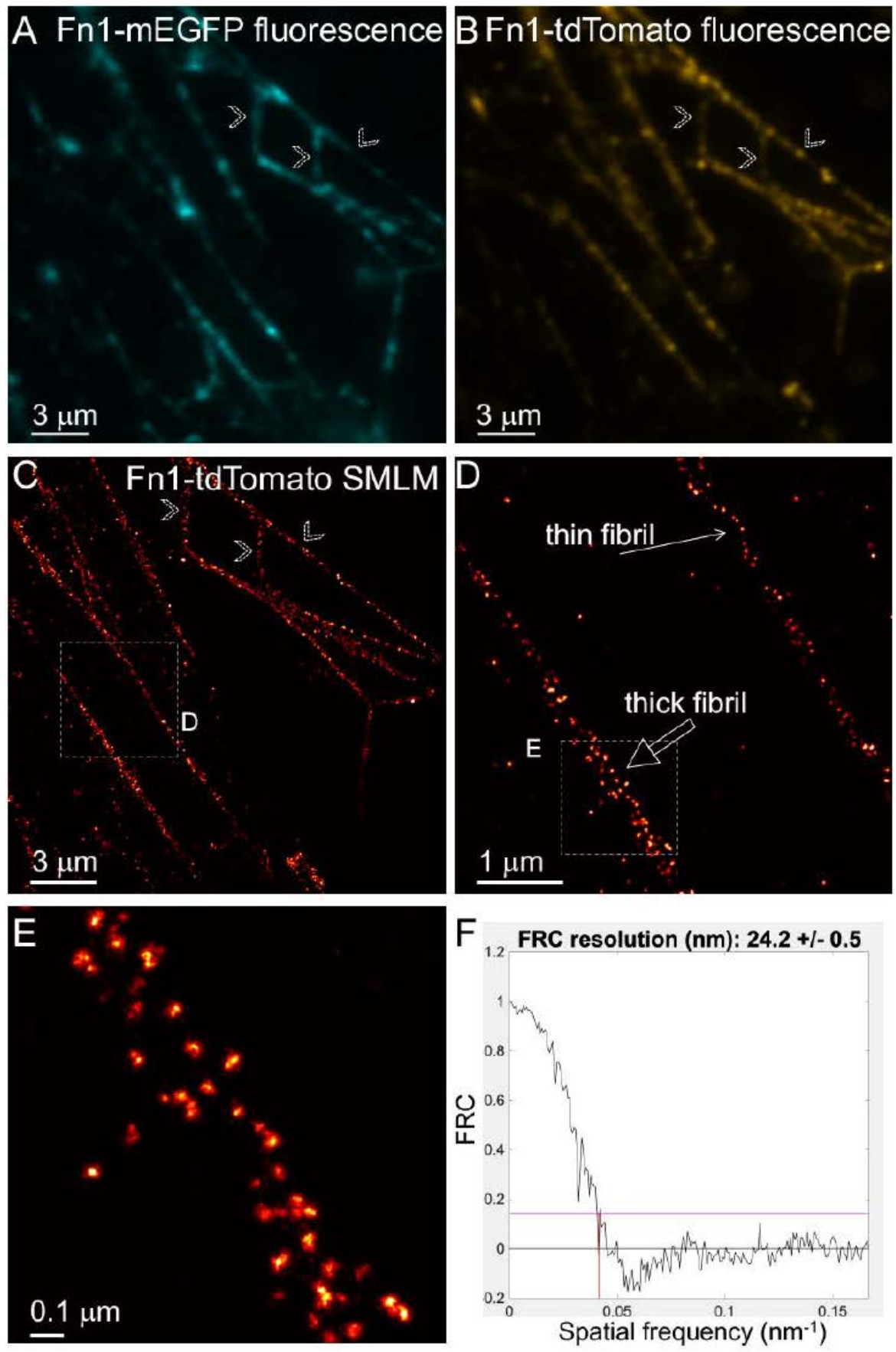


Fig. S7. Ectopically-added Fn1-tdTomato co-assembles with endogenous Fn1-mEGFP fibrils and forms nanodomains. *Fn1*$^{mEGFP/mEGFP}$ MEFs were plated on glass without coating at 50% confluency for 16 hours and then incubated with 10 μg of Fn1-tdTomato for 24 hours. Cells were then fixed and stained with rabbit anti-Cherry antibody, which was detected with secondary antibodies conjugated with Alexa-647. Fn1 ECM deposited between cells was imaged. **A** – **B.** Widefield images taken with 100X Objective, NA 1.49. **A.** Fn1-mEGFP fluorescence. **B.** Fn1-tdTomato fluorescence. **C** – **E.** dSTORM using SMLM protocol I. **C.** dSTORM image of the region shown in **B.** Examples of fibrils in **C** corresponding with fibrils in the wide-field images (**A-B**) are marked with chevrons. **D.** Magnified region containing a thick and a thin fibril seen in **C. E.** Nanodomain architecture of the thick fibril region boxed in **D.** Note nanodomain architecture of ectopically-added Fn1-tdTomato. **C-E** SMLM images were reconstructed using SMAP (see Methods). **F.** Fourier ring correlation (FRC) analysis was performed in SMAP. FRC curve shows the decay of correlation with increasing spatial frequency. Pink line marks the threshold value of 0.143 calculated for SMLM data (Nieuwenhuizen et al., 2013). The Red line marks the spatial frequency for which the threshold falls below the value of 0.143. Resolution is calculated as the inverse of the spatial frequency. The resolution of the region shown in **E** is 24.2+/-0.5 nm.

Supplemental Figure 8

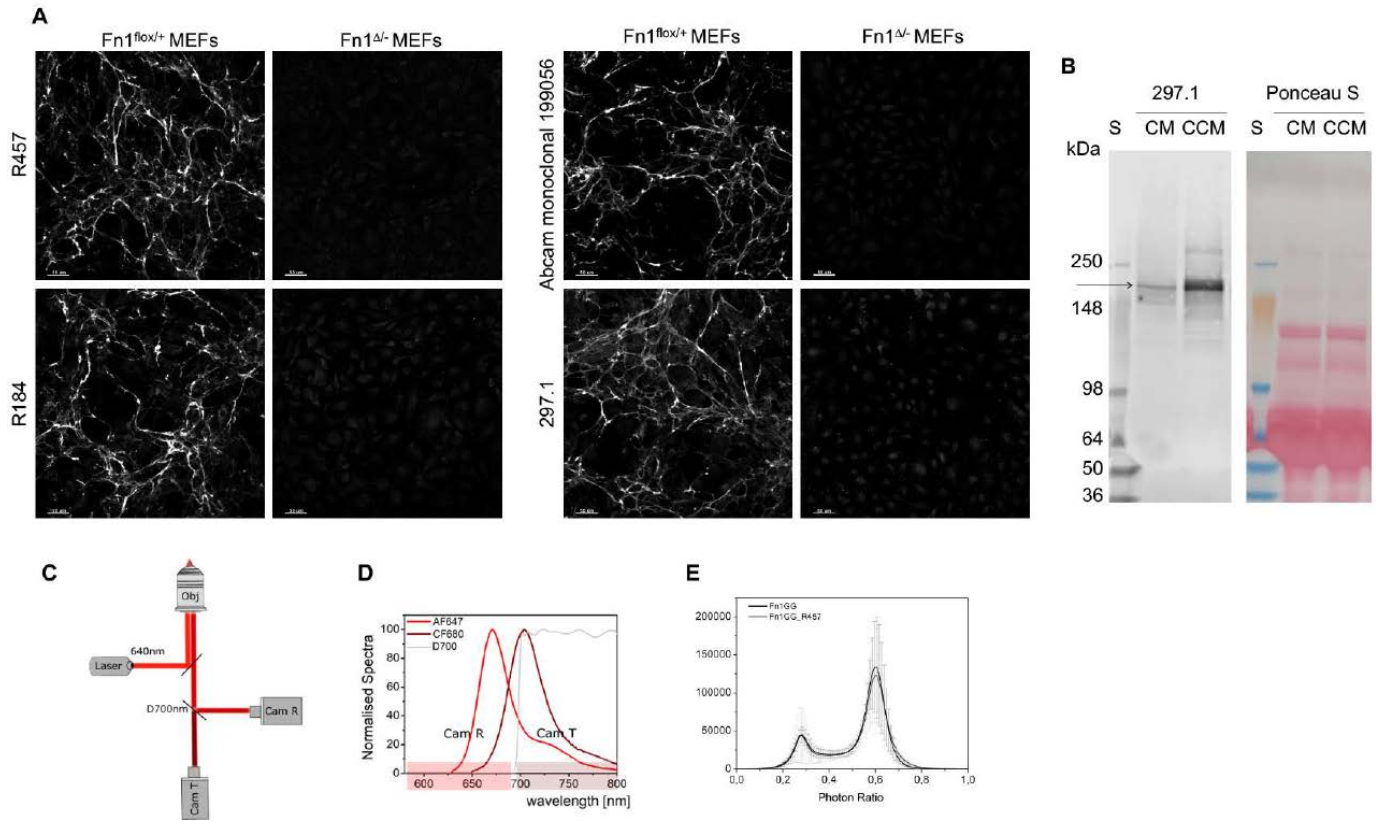


Fig. S8. Antibodies utilized in this study are specific to fibronectin. A. MEFs were isolated from $Fn1^{flox/+}$ or $Fn1^{flox/-}$ embryos at E14.5. $Fn1^{flox/-}$ MEFs were infected with adenoviruses encoding Cre recombinase and sorted two days after infection, generating $Fn1^{\Delta/-}$ MEFs lacking Fn1. Deletion of Fn1 was confirmed using western blotting (data not shown). $3x10^4$ cells per well were plated in a 24-well plate on 1.2 mm glass coverslips without coating and fixed with 4% PFA 72 hours later. Cells were stained with antibodies indicated to the left of the panels at 1:100 dilution. The same antibody solution was applied to $Fn1^{flox/+}$ and $Fn1^{\Delta/-}$ MEFs for each antibody. Primary antibodies were detected with anti-rabbit secondary antibodies conjugated with AlexaFluor-647 and imaged using identical settings. Signal intensity for stained Fn1 cells was increased when making these panels to show background fluorescence. **B. 297.1 antibody reacts with bovine Fn1.** CM – complete medium as in Table M1. CCM – conditioned complete medium. CCM was prepared by incubating $2x10^5$ wild-type MEFs with 2 ml of CM for 48 hours in one well of a 6-well plate. An equal amount of medium was loaded in wells of Novex™ WedgeWell™ 4 to 12% Tris-Glycine protein gel. Arrow points at the Fn1 band. **C-D. Spectral de-mixing optical scheme. C.** A 640 nm laser is sent through the objective lens to excite AF647 and CF680 fluorophores. The

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emitted light is separated by a 700 nm long pass dichroic filter that reflects light below 700 nm into the Reflected camera (Cam R) and transmits light above 700 nm into the Transmitted camera (Cam T). **D.** Emission spectra of AF647 and CF680 dyes together with a 700nm dichroic filter showing spectral separation into the two cameras Cam R and Cam T. **E.** Average ratio distributions for *Fn1*$^{mEGFP/mEGFP}$ cells stained using chicken anti-GFP and rabbit anti-GFP antibodies (Fn1GG, dark gray) or chicken anti-GFP and rabbit R457 antibodies (Fn1GG_R457, lighter gray). The two fluorophore populations can be clearly distinguished for lambda assignment.

Table S1. Effective labeling efficiency and standardized SMLM measurements

Sample	Buffer	ELE (%)	Average # Localizations per NPC	# Localizations per protein	Radius of NPC, nm
U2OS-Nup96	GLOX/BME	79.6+/- 5.4	280 +/- 49.2	8.75 +/- 1.54	63.6 +/- 0.86

NUP96$^{mEGFP/mEGFP}$ cells were imaged using αGFP 1° antibody and AF647-conjugated 2° antibody detecting NUP96-mEGFP; SMLM imaging protocol I was used to acquire data. Data is from 3 independent experiments, eight cells, and 4571 NPCs

Table S2. Characterization of Fn1-mEGFP nanodomains in Fn1 fibrils using standardized SMLM protocol

	Buffer	Average PSF (nm)	Average # Localizations per nanodomain	Median Localization Precision, nm	# of mEGFP molecules per nanodomain in Fn1-GFP fibrils	Apparent Diameter of Fn1 nanodomain, nm	Nanodomain periodicity, nm
Fn1$^{mEGFP/mEGFP}$ MEFs; αGFP 1° antibody; AF647-conjugated 2° antibody	GLOX/BME	143.8 +/- 2.35	159 +/- 77.92	181	16.85 +/- 5.1	28.26+/-9.7	102±29

αGFP 1° antibody; AF647-conjugated 2° antibody were used to detect Fn1-mEGFP. SMLM imaging protocol I was used to acquire data. Data is from 3 independent experiments, 6 cells, 52 fibrils and 833 nanodomains were used to calculate the number of GFP molecules per nanodomain; To quantify nanodomain dimeter, data from 3 independent experiments, 15 cells, 27 fibrils, and 1292 nanodomains were used.

Table S3. Cell Culture Media

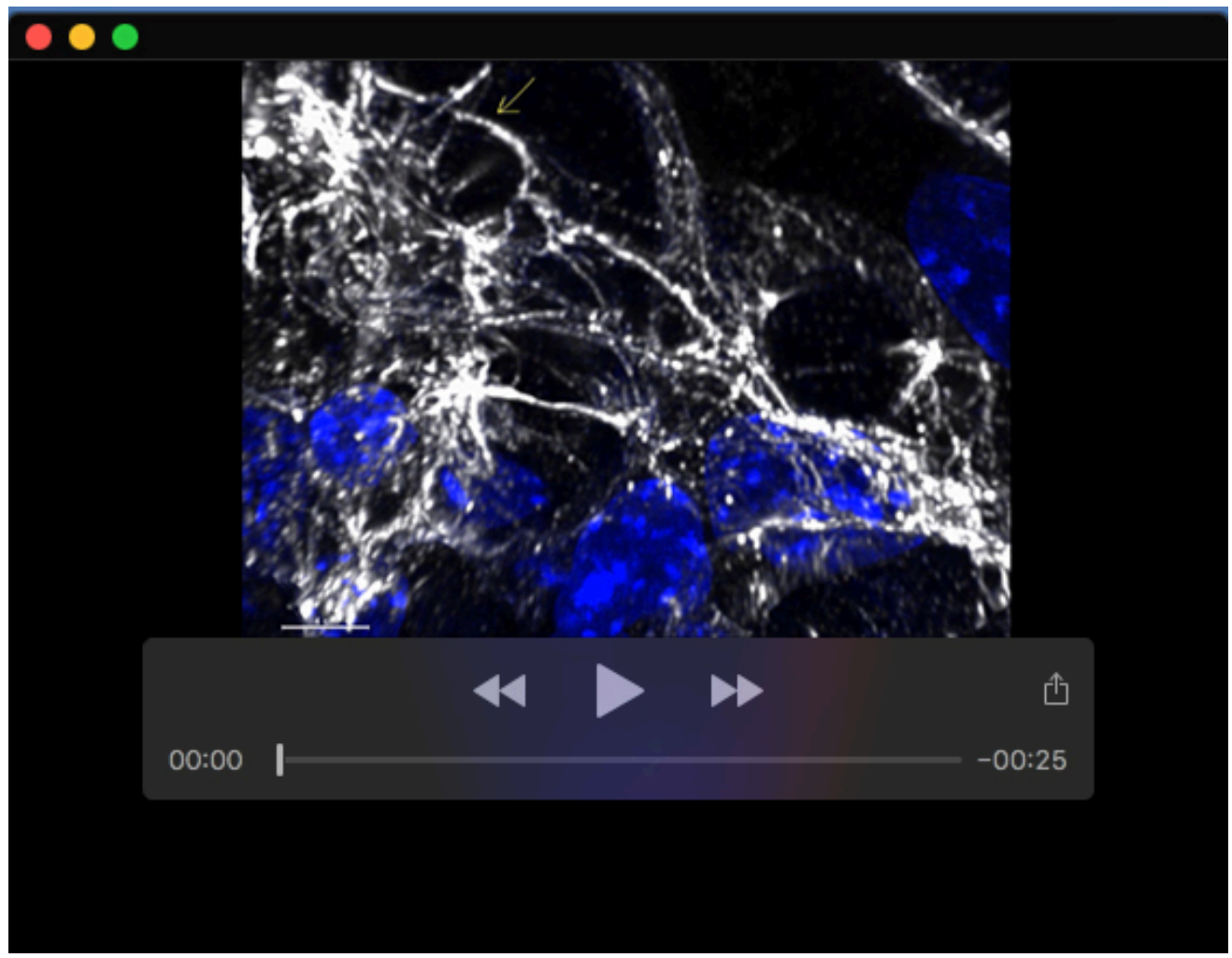
	Cells	Composition of Medium
Complete medium	MEFs	High-glucose Dulbecco's Modified Eagle Medium (DMEM, Corning, cat # 10-013-CV) supplemented with 10% v/v fetal bovine serum (Gemini Biosciences, cat # 100-106), 1% v/v penicillin/streptomycin solution (GE Healthcare, cat #SV30010), 1% v/v L-glutamine (Gibco, cat # 35050-061).
Imaging medium	MEFs	FluoroBrite DMEM (Thermo Fisher Scientific, catalog # A1896701) supplemented with 2% v/v fetal bovine serum (Gemini Biosciences 100-106), 1% v/v penicillin/streptomycin solution (GE Healthcare, SV30010), 1% v/v L-glutamine (Gibco 35050-061), pH8.14.
McCoy's 5a	Nup96-mEGFP U2OS cells	McCoy's 5a medium (VWR, cat # MSPP-302007) containing 1xGlutaMax (ThermoFisher, cat #35050061), 10% v/v fetal bovine serum (Gemini Biosciences, cat # 100-106), and 1x penicillin/streptomycin (GE Healthcare, cat #SV30010).

Table S4. Antibodies

Primary Antibodies (1° Ab)	Specificity	Source/ reference
Abcam recombinant rabbit monoclonal	Epitope recognized by this antibody is located within 301 aa between amino acids 1350- 1651 of mouse Fn1, transcript variant 2, mRNA, RefSeq NM_001276408	Abcam, cat # 199056
297.1 rabbit polyclonal	Raised to full-length rat plasma Fn1. Recognizes multiple epitopes, see **Fig. S3**	Richard Hynes lab (Rickelt and Hynes, 2018)
R457 rabbit polyclonal	70 kDa N-terminal domain	Jean Schwarzbauer's lab (Aguirre et al., 1994; Sechler et al., 2001)
R184 rabbit polyclonal	First six type III repeats of Fn1	Jean Schwarzbauer's lab (Raitman et al., 2018)
3E2 mouse monoclonal	EIIIA	Sigma, cat #SAB4200784-100UL
GFP, chicken polyclonal	GFP	Aves, cat# GFP-1010;
GFP, rabbit polyclonal	GFP	MBL International; cat #598
Itgα5, rat monoclonal	Integrin alpha 5	BD biosciences, cat # 553319
mCherry, rabbit polyclonal	Cherry; tdTomato is composed of two mCherry molecules in tandem (Shaner et al., 2005)	Abcam, cat # ab167453

Secondary Antibodies (2° Abs)	Source, catalog #, concentration	Dilution
AffiniPure F(ab')$_2$ Fragment Donkey Anti-Rabbit IgG (H+L)	Jackson Immunoresearch, cat # 711-606-152, 1.5 mg/ml	1:300
Donkey anti-Mouse IgG (H+L) Highly Cross-Adsorbed Secondary Antibody, Alexa Fluor 555	ThermoFisher Scientific, A-31570, 2 mg/ml	1:300
Cy™3 AffiniPure F(ab')$_2$ Fragment Donkey Anti-Rat IgG (H+L)	Jackson Immunoresearch, 712-166-150, 1.5mg/ml	1:300
Alexa Fluor® 488 AffiniPure F(ab')$_2$ Fragment Donkey Anti-Chicken IgY (IgG) (H+L)	Jackson Immunoresearch, 703-546-155, 1.5mg/ml	1:300

Alexa Fluor® 647 AffiniPure F(ab')$_2$ Fragment Donkey Anti-Chicken IgY (IgG) (H+L)	Jackson Immunoresearch, 703-606-155, 1.5mg/ml	1:300
CF680 Donkey anti-rabbit, highly cross-adsorbed	Biotium, cat # 20418-50ul), 2mg/ml	1:200



Movie 1. Rotational views through the Fn1+ ECM in the cardiac jelly. The whole E9.5 embryo was stained using Abcam rabbit monoclonal anti-Fn1 antibody and imaged using 100x objective, N.A. 1.49, with the pinhole set at 0.8 Airy units, and sampling of 40 nm per pixel in x, y. The movie shows 3D reconstruction through 3.4 μm of tissue sampled every 0.121 μm in z. Fn1 is in white, DAPI is in blue. Arrows point to examples of beaded Fn1 fibrils.

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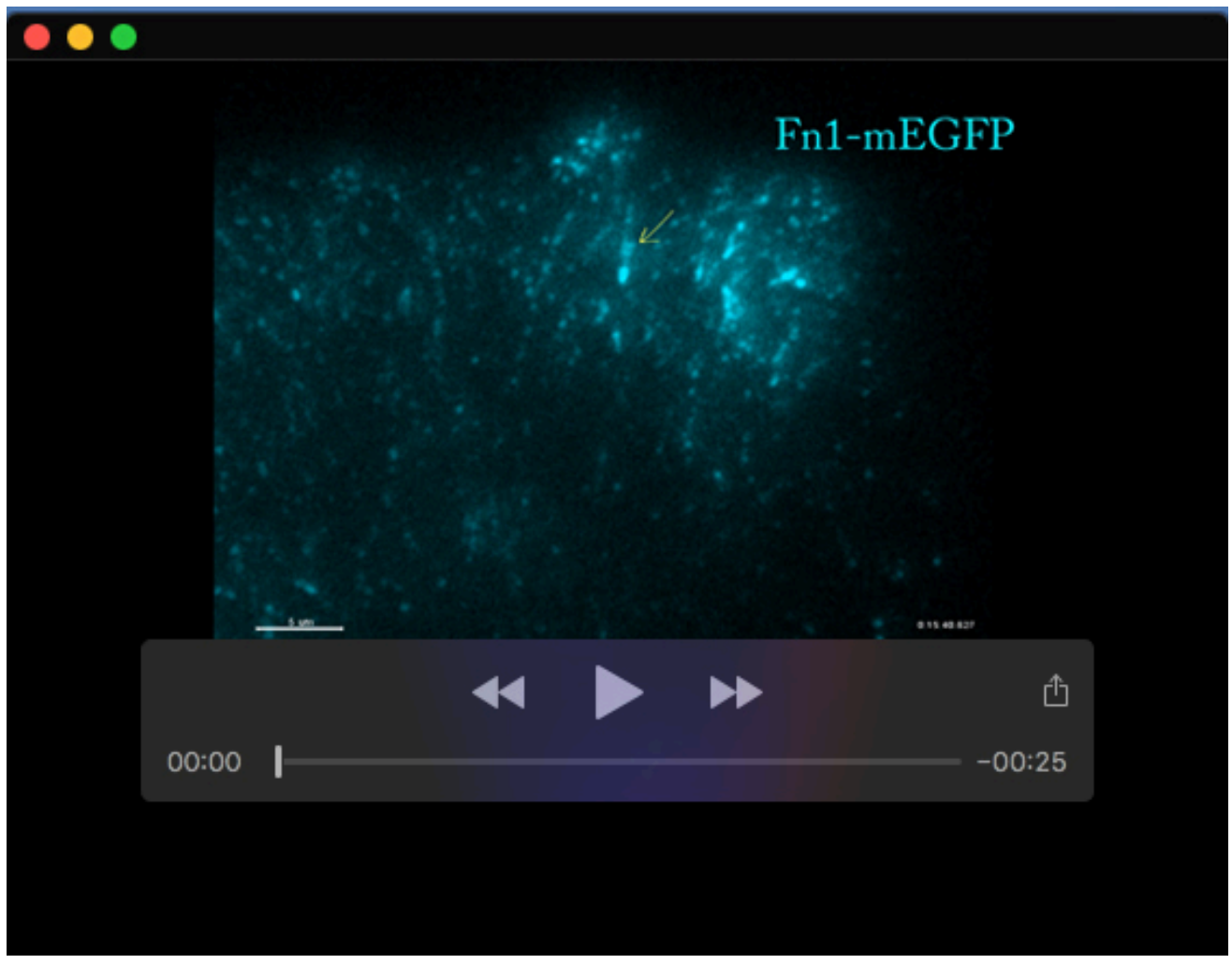
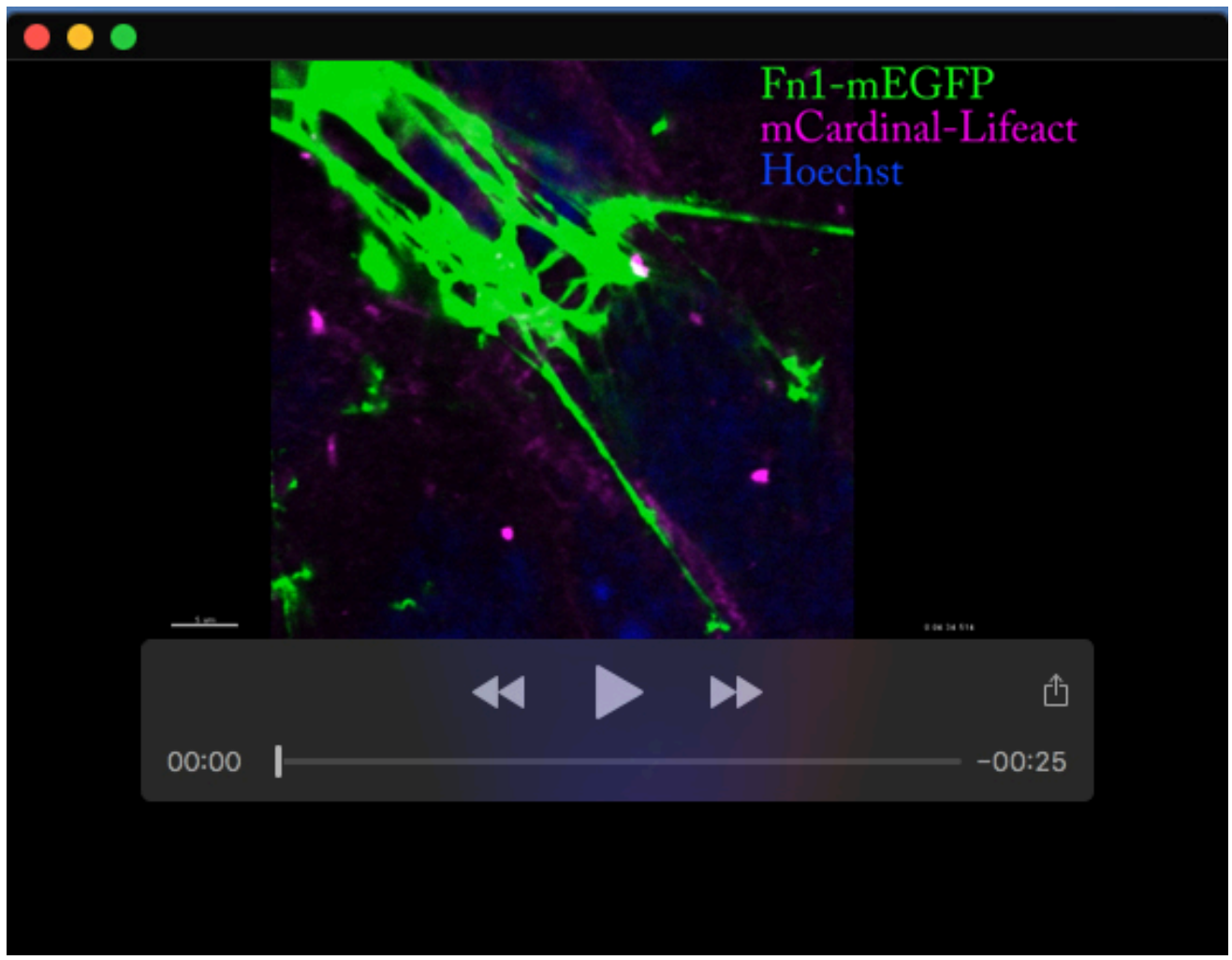
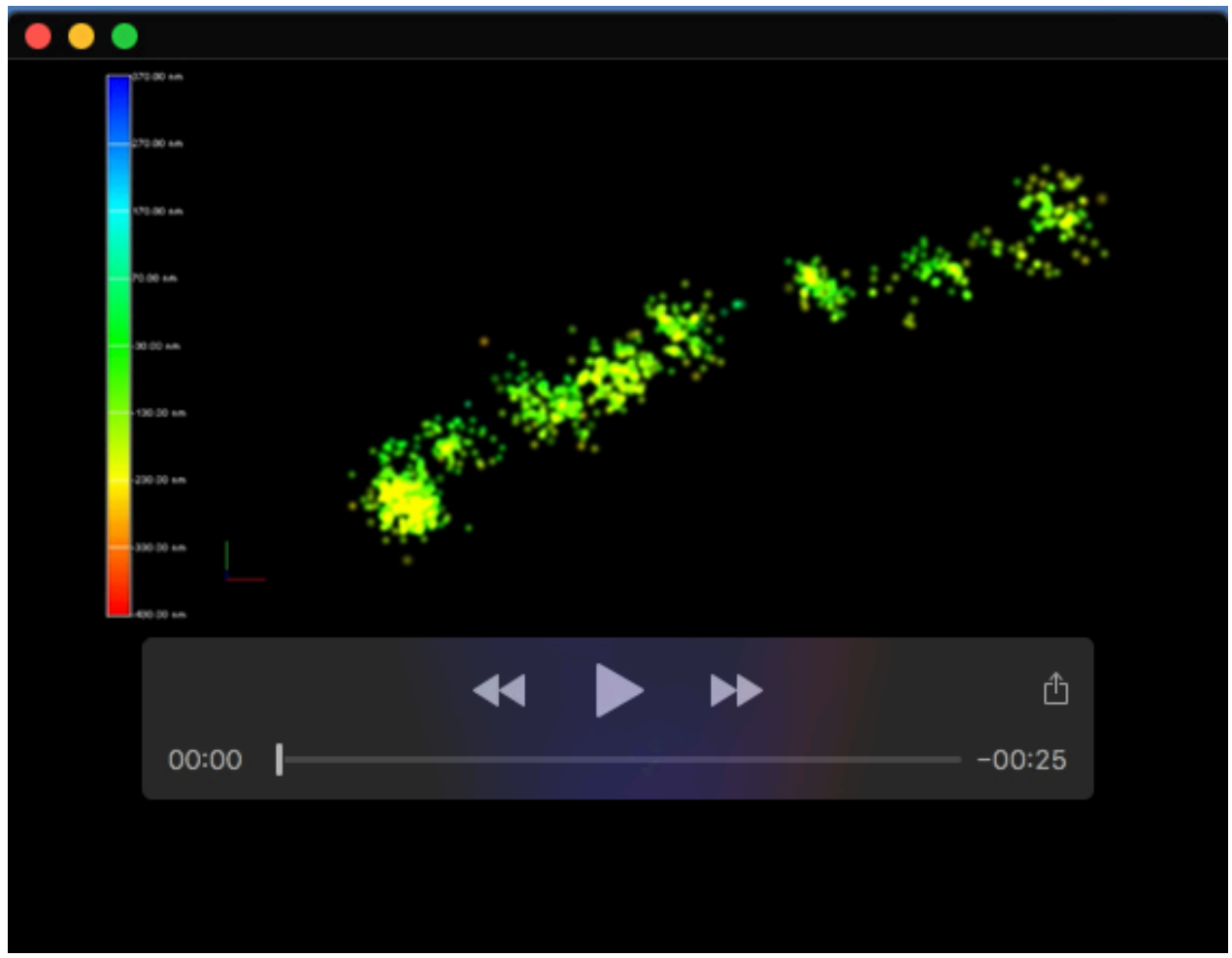


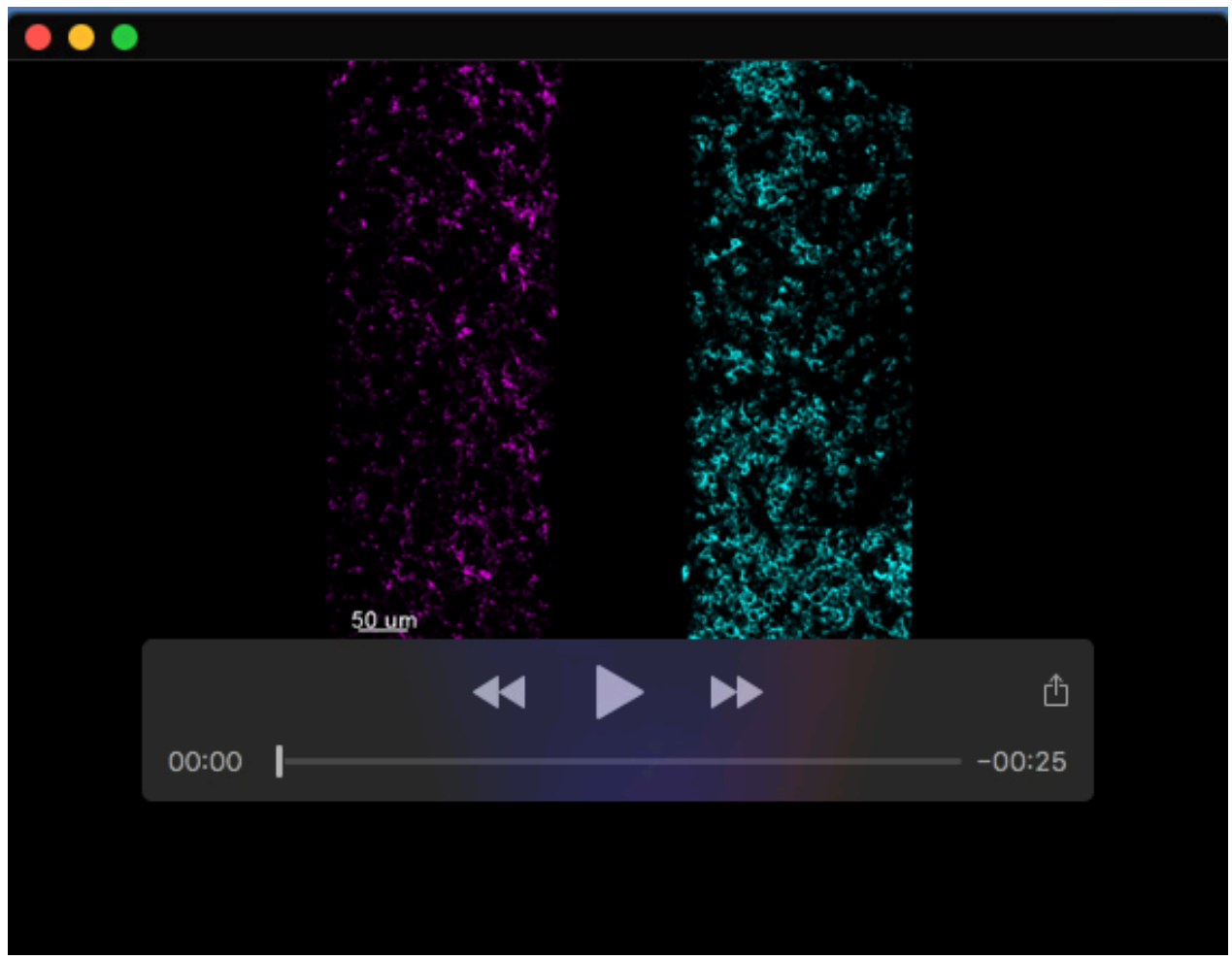
Movie 2. Fn1 fibrillogenesis imaged by TIRF microscopy. Fn1^{mEGFP} MEFs were transiently transfected with mCardinal-Lifeact, plated on gelatin-coated glass coverslips, and imaged 48 hours later. Filming was done every 2 min for 30 min using TIRF and 100x objective, N.A. 1.49. The first set shows the Fn1-mEGFP channel. Yellow arrows point to centripetally-moving Fn1 nanodomains organized into an elongating linear fibril. The second set is an overlay between Fn1-mEGFP and mCardinal-Lifeact.



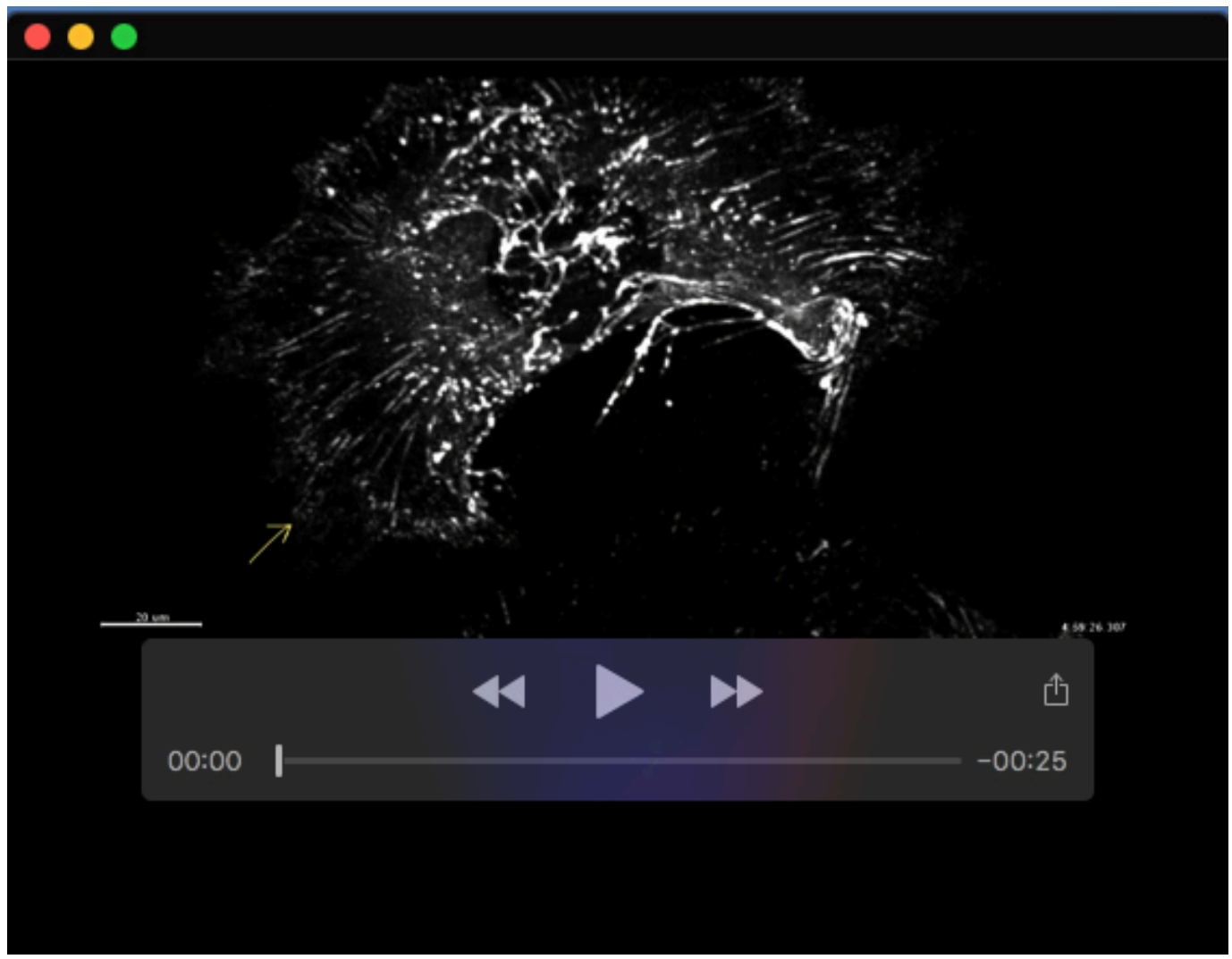
Movie 3. 2% DOC dissolved cytoplasm and nucleus in under 13 min leaving cell-free Fn1 fibrils. MEFs expressing Fn1-mNeonGreen were plated on glass-bottom slides without coating and labeled with SiRActin (magenta) to visualize F-actin, and Hoechst (blue) to visualize DNA. Time-lapse was recorded every 54 sec immediately following the addition of the DOC solution pH 8.01 to live cells. The presence of 2% DOC dissolves actin cytoskeleton and nuclei and leaves cell-free Fn1 ECM fibrils (green). Fn1 fibrils collapse following the dissolution of the actin cytoskeleton due to the loss of tension.



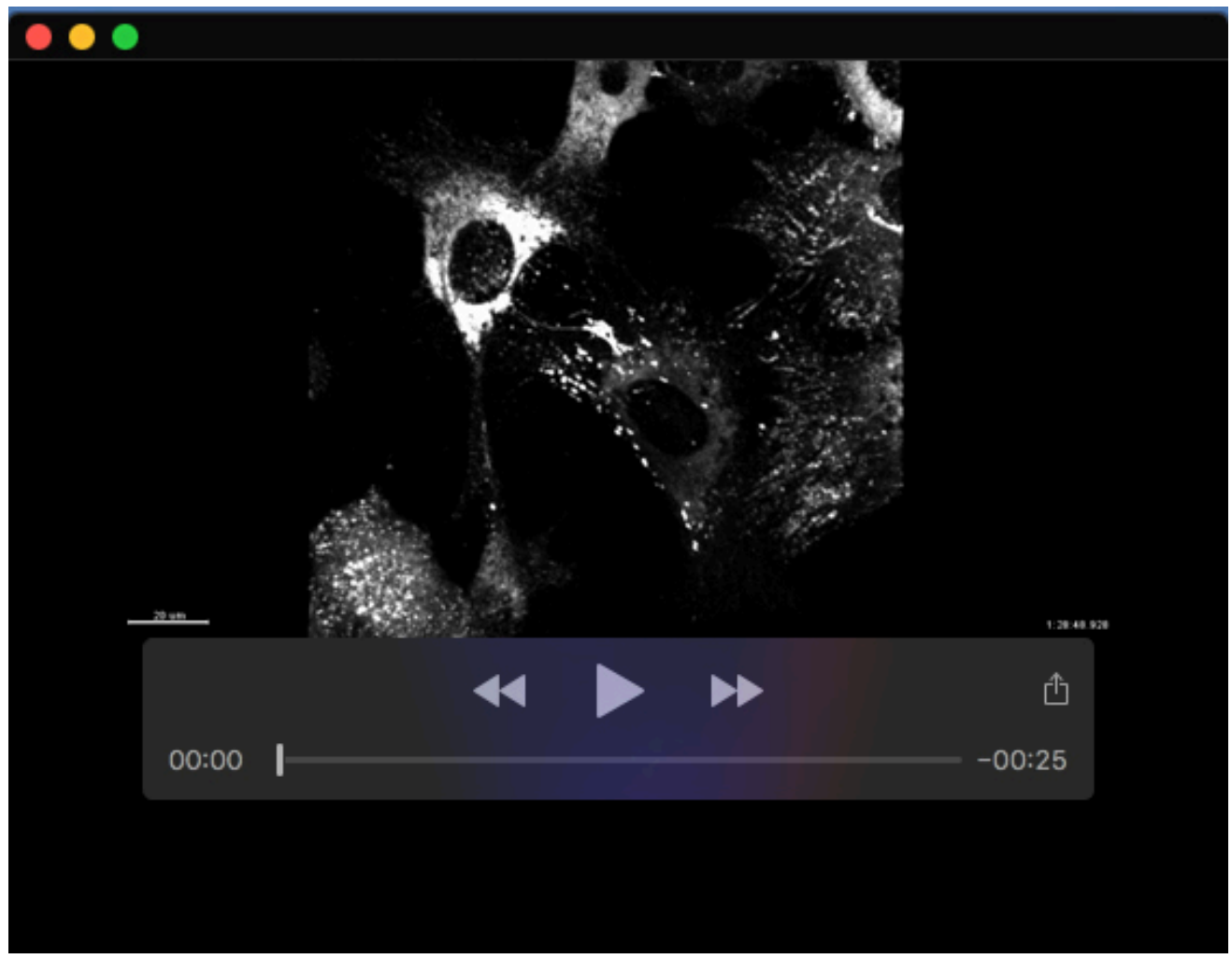
Movie 4. 3D-rendering of STORM data. Fn1 fibril detected using anti-GFP antibodies is rotated around the x-axis to show the arrangement of Fn1 localizations in 3D. The movie starts in x-y plane. Yellow arrows point at Fn1 nanodomains in the fibrils. Red arrows point to the space between the nanodomains.



Movie 5. Fibrillogenesis of ectopic Fn1. 3-well culture insert was placed in the middle of the 35 mm glass-bottom dish and 0.8 x 10^6 Fn1-tdTomato-expressing MEFs were plated surrounding the inserts. 24 hours later, Fn1-mEGFP-expressing MEFs were plated inside the inserts on glass without coating for 5 hours. Confocal live imaging of areas containing Fn1-mEGFP-expressing MEFs was performed using Plan Fluor 40x Oil (NA 1.3). Positions containing Fn1-mEGFP-expressing MEFs were imaged at 17-18 min intervals for ~16 hours. This movie contains maximum intensity projections composed of forty-three confocal slices at 0.5 μm thickness, the pinhole was set to 1 Airy unit. The first still panel in this movie is a montage of the plate to show Fn1-tdTomato and Fn1-mEGFP-expressing cells prior to the start of the time-lapse.



Movie 6. Cells incubated with III-11C, show robust fibrillogenesis. *Fn1*$^{mEGFP/+}$ MEFs were plated on uncoated glass in 8-well Ibidi chambers for 4 hours. Medium containing 11-IIIC control peptide was then added and cells were filmed every 90 sec for about 15 hours, as described in Methods. The movie begins approximately 30 min after the 11-IIIC-containing medium was added, the time it takes to set up a time-lapse recording. Arrows point to the cell periphery and examples of centripetally moving Fn1 fibrils.



Movie 7. FUD interferes with the linking of centripetally moving Fn1 nanodomains into fibrils. Fn1$^{mEGFP/+}$ MEFs were plated on glass in 8-well Ibidi chambers for 4 hours. Medium containing FUD peptide was then added and cells were filmed every 3 min for about 15 hours, as described in Methods. The movie begins approximately 30 min after the FUD-containing medium was added, the time it takes to set up a time-lapse recording. Note the dismantling of the remaining pre-existing fibrils at the beginning of the movie. Yellow and red arrows point to the cell periphery. Note the presence of centripetally moving Fn1-mEGFP “beads” and the scarcity of Fn1 fibrils for the majority of the duration of the movie.