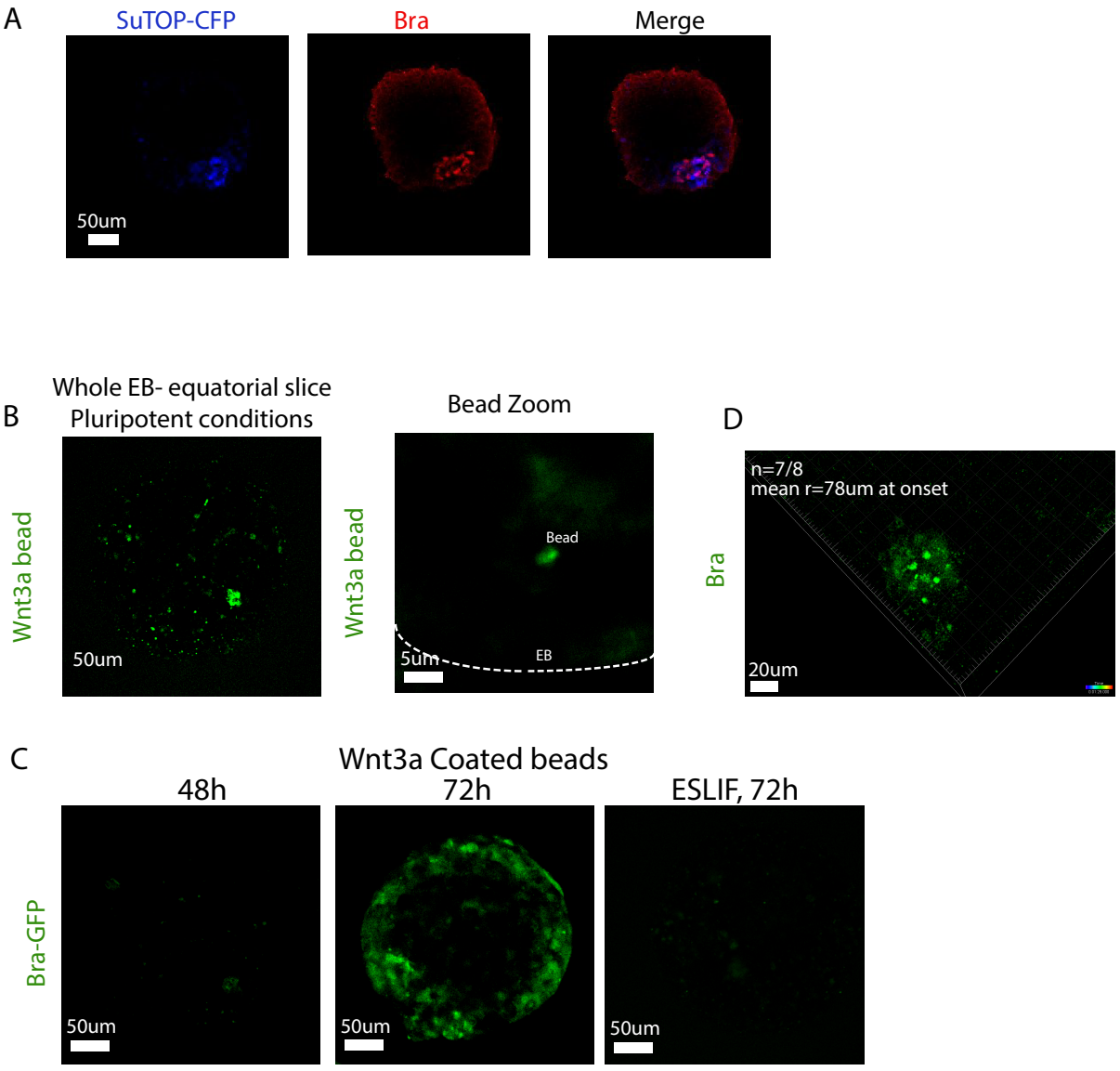


Fig S1. Wnt3a beads integration and small-EB isotropic onset. (A) Bra co-localizes with canonical Wnt response in SuTOP-CFP cells. (B) Left: An equatorial slice of an EB embedded with multiple Wnt3a-coated beads (green). Right: A zoom in snapshot on a Wnt3a-coated bead. (C) Wnt3a-coated embedded beads have no effect on Bra onset in pluripotent conditions (right) or in early differentiation (left), however onset is isotropical on the shell at 72 hrs. (D) An example small EB differentiated in microwells at 72 hrs from aggregation, showing isotropic (spatially uniform) Bra expression onset. The isotropic pattern occurred in 7/8 small EBs (radius at onset = 78+-10um)

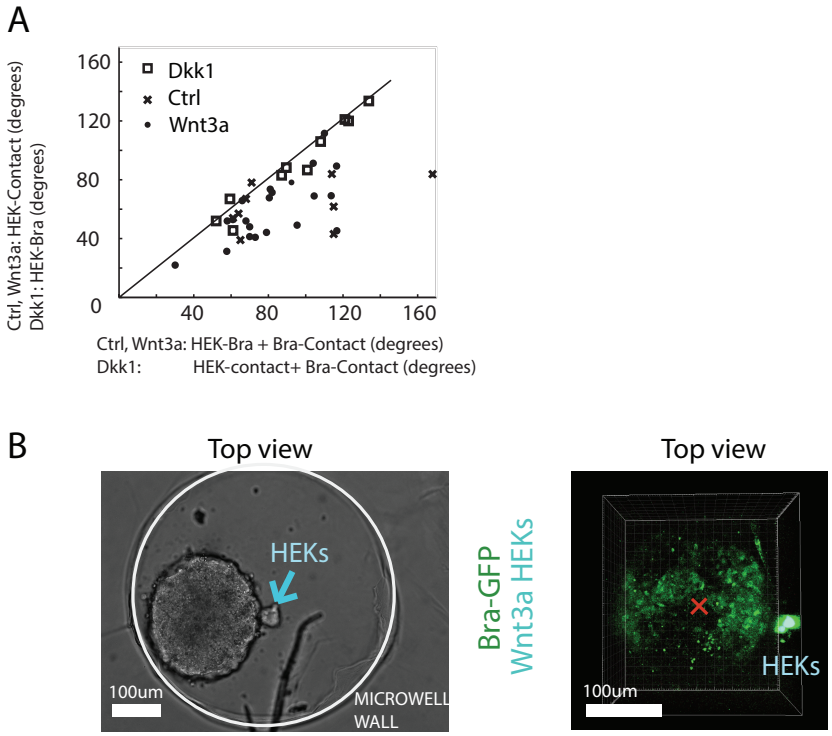


Fig S2. Wnt3a/Dkk1 source, Bra locus, and contact point show high co-planarity. (A) The angle between HEKs signal source and contact (δ, Y-axis) vs. the sum of the angle between HEKs to Bra locus and the angle between Bra locus and contact ($\theta+\varphi$, X-axis) for all EBs quantified in Fig. 4E,F. For EBs harboring Dkk1 or Wnt3a producing HEKs, δ is approximately equal to $\theta+\varphi$, indicating that Bra locus inhabits the same plane defined by the HEKs, contact point and the EB centroid. As expected, in control EBs there is a larger deviation from co-planarity, as the Bra locus is not constrained to that same plane. (B) An example of double Bra loci, one triggered by localized Wnt signaling and the other from contact with the microwell wall.

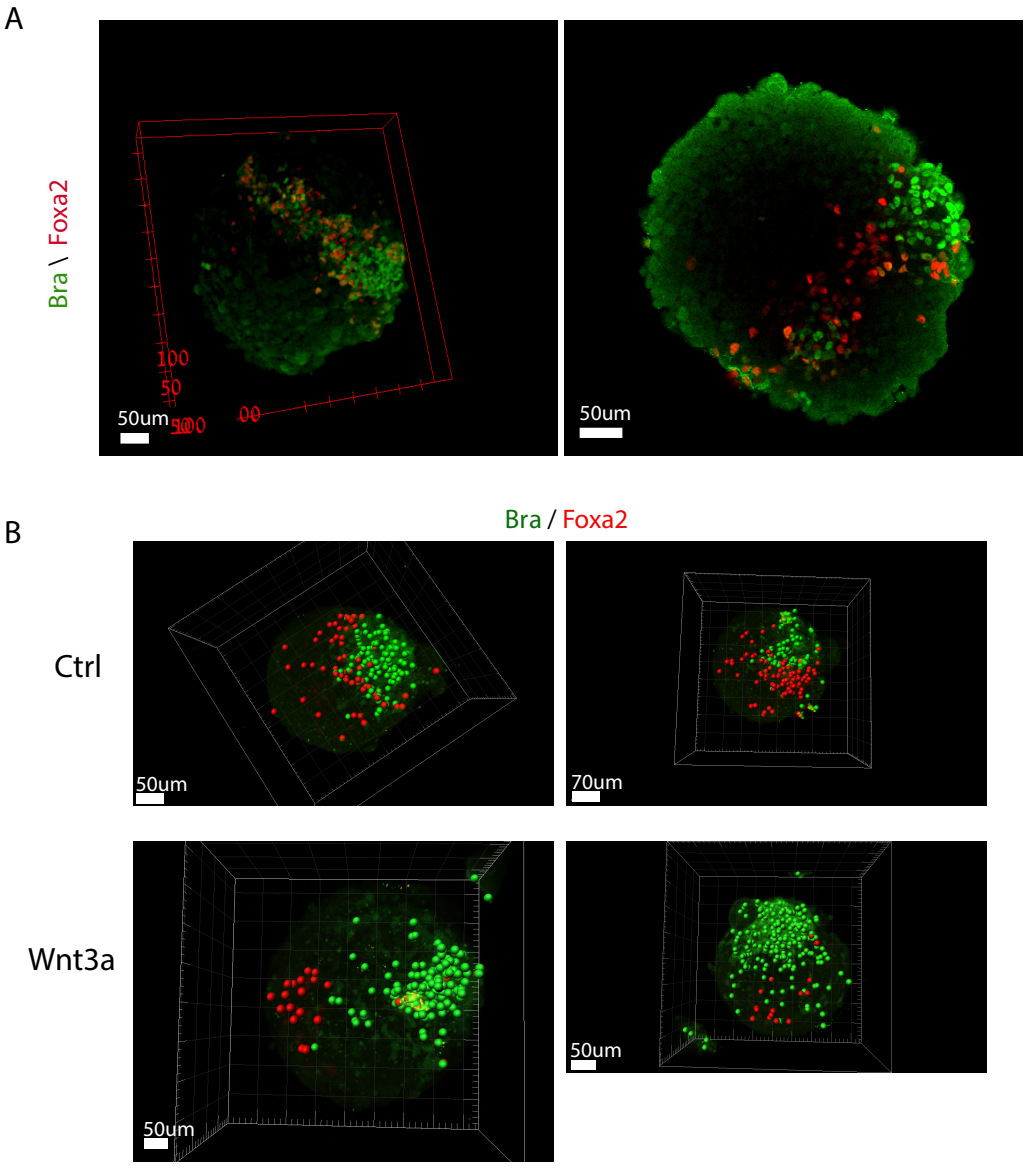


Fig S3. Bra and Foxa2 are spatially adjacent in EBs but can be decoupled by external Wnt signaling. (A) Immunostaining of Foxa2 and Bra at 72 hrs from aggregation. Foxa2 spatially correlates with Bra expression. (B) Segmentation of immunostained Foxa2 (red) and Bra (green) expressing cells at 72 hrs from aggregation. Foxa2 is adjacent to Bra locus in wild type EBs, while Wnt3a treated EBs show Foxa2 downregulation and spatial decoupling from Bra.

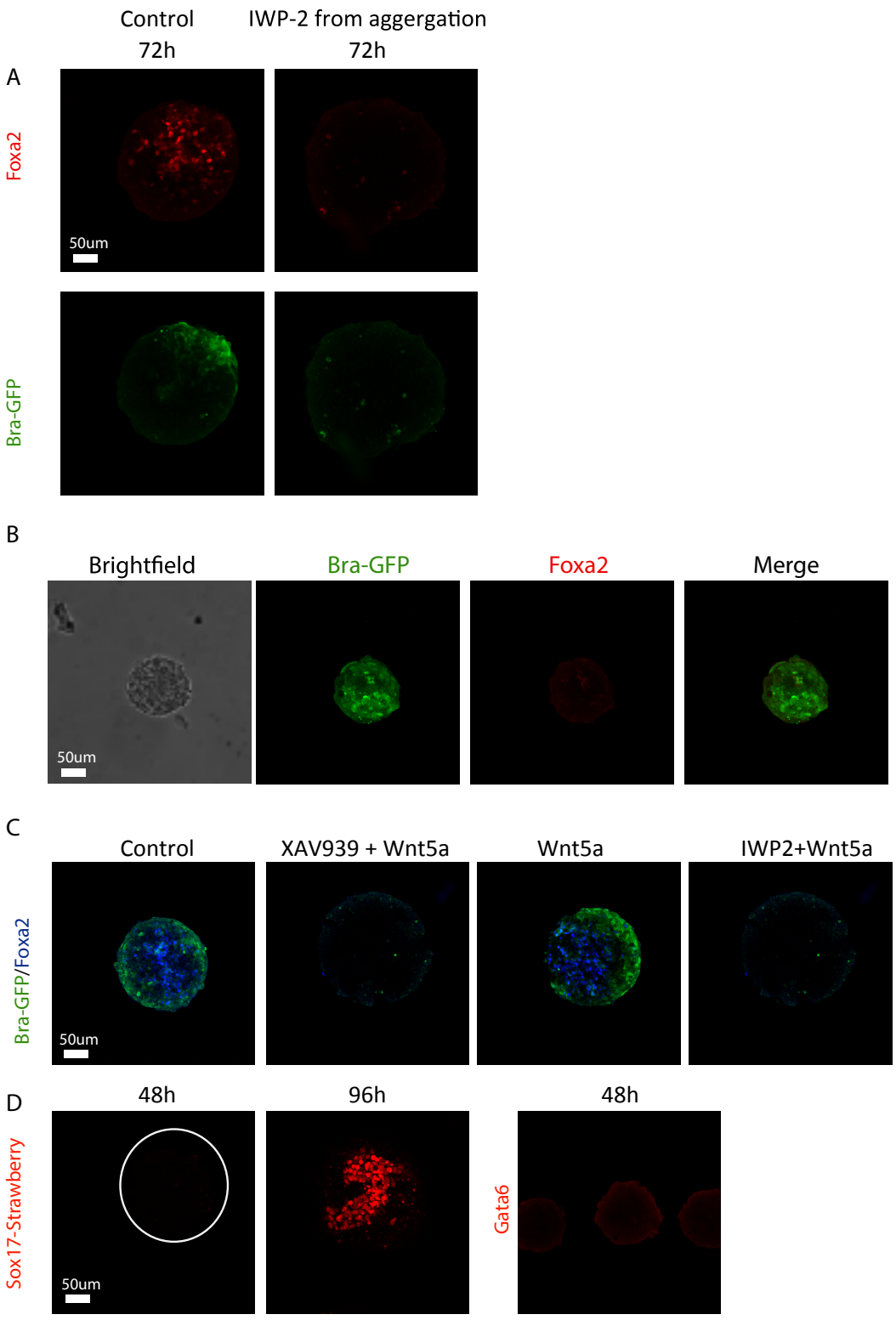
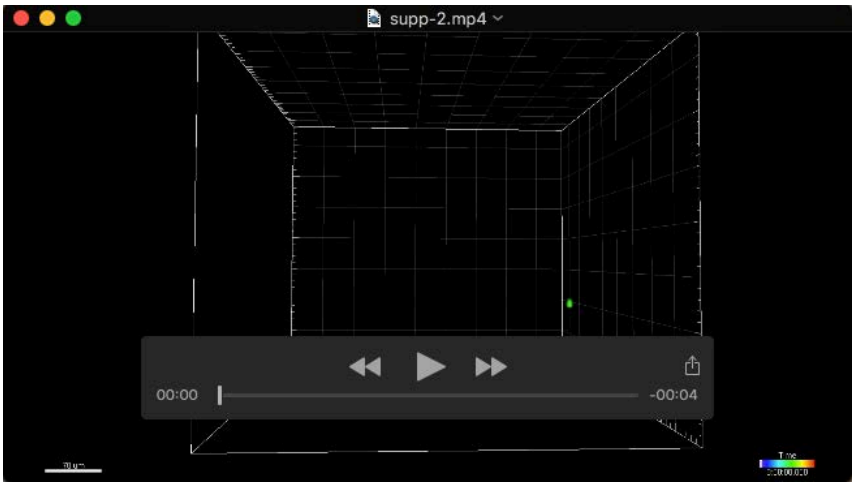
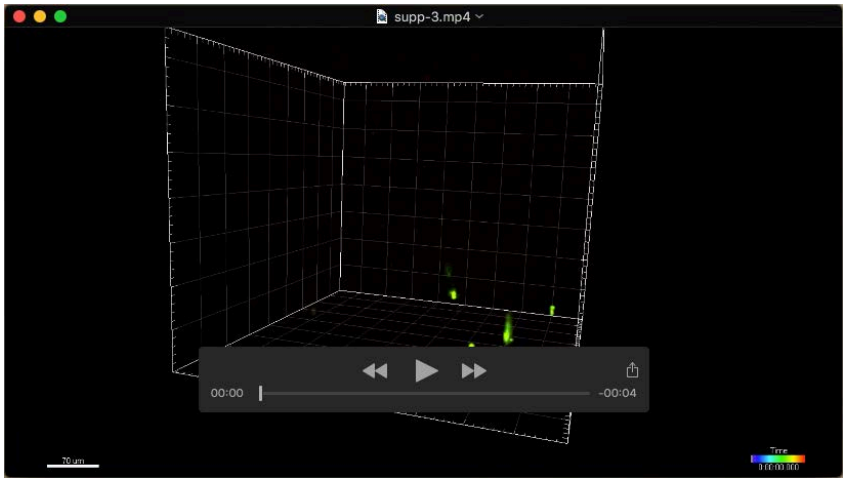


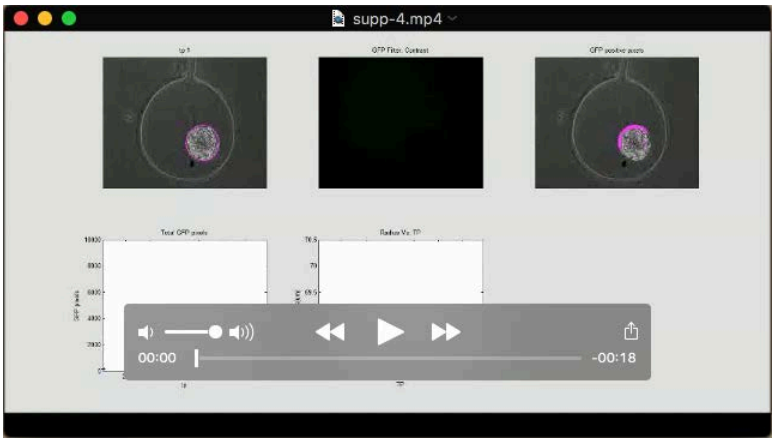
Fig S4. Foxa2 expression abolished under IWP-2 treatment or in small sized EBs. (A) Bra and Foxa2 are not expressed at 72 hrs under IWP-2 perturbation starting at 0 hrs from aggregation. (B) Small size EBs do not express Foxa2 at 72 hrs. (C) Foxa2 and Bra are not expressed under canonical Wnt inhibition (XAV939) or general Wnt inhibition (IWP2), and are not rescued by non-canonical Wnt activation (Wnt5a). (D) Sox17 and Gata6 are not expressed before Bra onset, indicating Foxa2+ cells at 48 hrs do not represent an endodermal lineage.



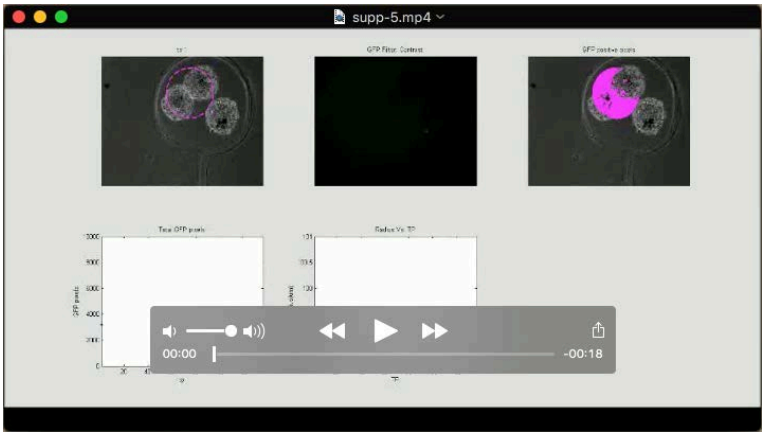
Movie 1 Three-dimensional time-lapse imaging of Bra-GFP in two E14 Bra-GFP embryoid bodies, imaged in microwells between 60 and 96 hrs from aggregation and transfer to differentiation medium. Bra-GFP expression onsets at the bottom (contact point with the glass) , and expands upwards on the outer shell.



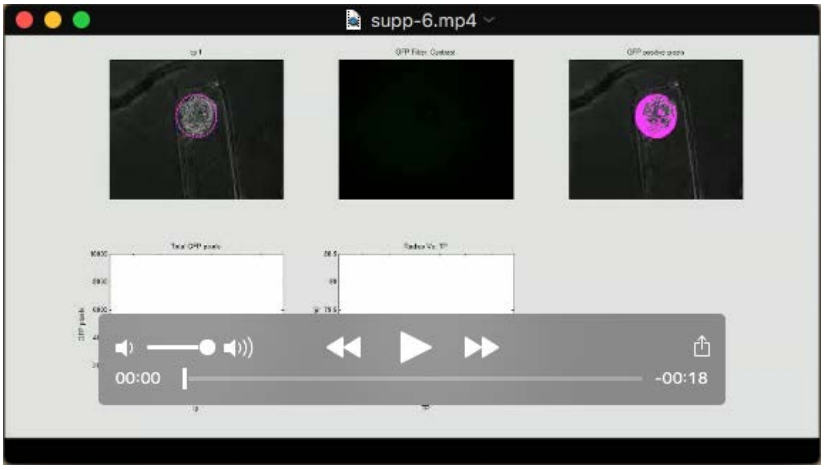
Movie 2 Three-dimensional time-lapse imaging of Bra-GFP in two E14 Bra-GFP embryoid bodies, imaged in microwells between 60 and 96 hrs from aggregation and transfer to differentiation medium. Bra-GFP expression onsets at the bottom (contact point with the glass) , and expands upwards on the outer shell.



Movie 3 Epifluorescence time-lapse of a Bra-GFP embryoid body, where Bra onset occurred at the bottom. The EB is imaged between 24 and 90 hrs from aggregation, where at 24 hrs it was transferred to a microwell. Top row: left - brightfield imaging of the EB with its encompassing perimeter; center - Bra-GFP; right – overlay of brightfield and GFP-positive pixels (magenta) . Bottom row: left - total Bra-GFP+ pixels vs. time point (time interval between points – 30 minutes) . Blue line - raw data; red dashed line: alpha filter smoothing; yellow line: Bra onset threshold defined as 500 GFP+ pixels; center – EB radius vs. timepoint. Noise at higher time points is due to manual estimation of radius; right – snapshot of Bra-GFP onset frame.

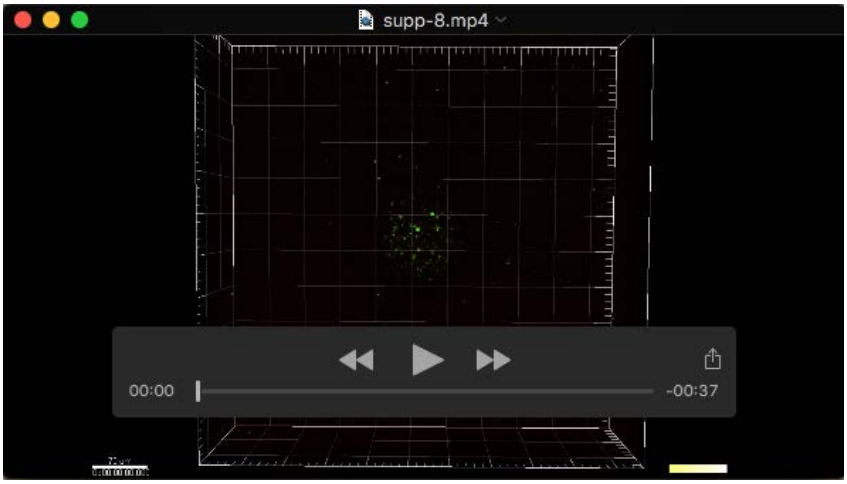


Movie 4 Similar to Movie S3, for a case where Bra expression onset occurred at the contact point with the microwell side. Note in this case the EB originated from 3 smaller EBs that merged together after transfer to the microwell.

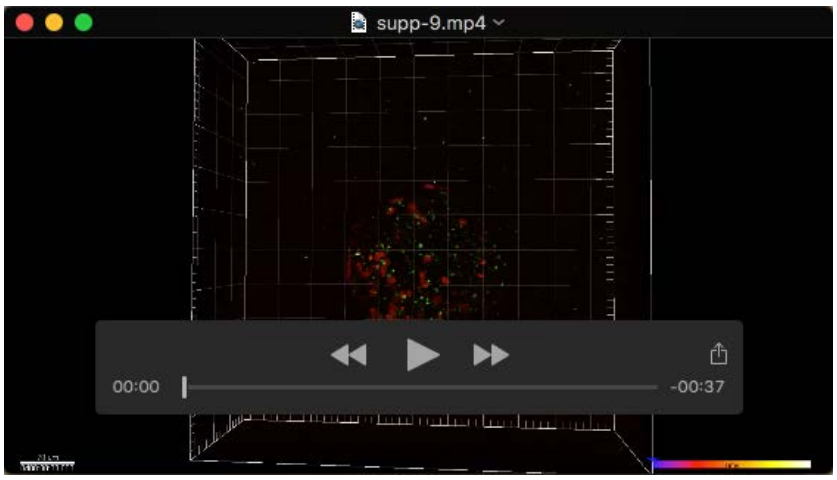


Movie 5 Similar to Movie S3, for an EB differentiated in an elongated micropool (width=200um). In this case, Bra-GFP expression onsets from two different loci (around t.p. 121), at the two sides compressed against the well walls. At t.p. 130 the EB pops out of the channel, resulting in a change in its focus.

Movie 6 Similar to Movie S5, for a case where Bra expression onsets from one of the compressed sides of the EB.



Movie 7 Time lapse of an EB differentiated while embedded in Matrigel. Bra onset occurs uniformly from the whole sphere, representative of the dynamics in 13/15 imaged EBs.



Movie 8 A Bra-GFP, pCMV-Strawberry large EB differentiated while embedded in Matrigel. Bra expression onsets from one locus, expanding from that point into the whole sphere. This dynamic represents 2/15 imaged EBs.