

**Fig. S1. Single particle analysis of IFT and BBSome tip turnaround**

**A)** Distribution of the minimum dwell times of the IFT proteins in tip-FRAP experiments.

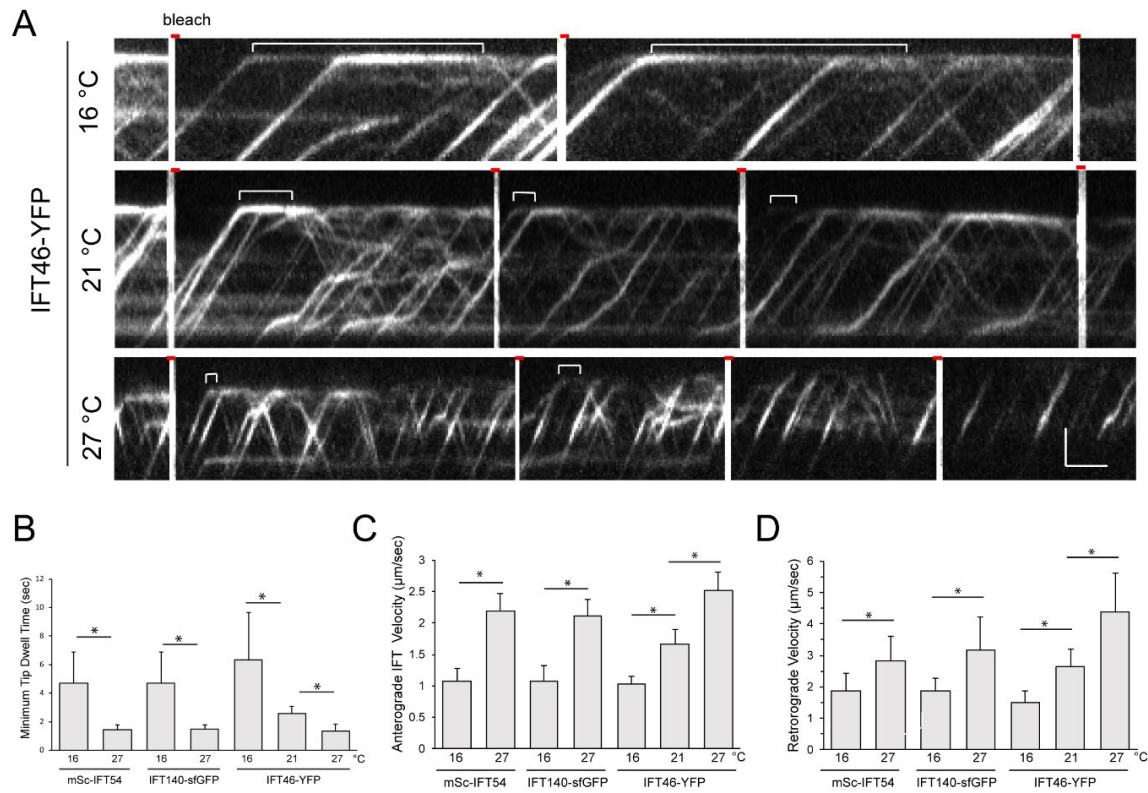
**B)** Kymograms showing the inactivated (Dendra<sup>GREEN</sup>) and activated (Dendra<sup>RED</sup>) form of 2xDendra-IFT54 expressed in the *ift54-2* mutant. a-c) Note the differences between the bulk flow of inactivated 2xDendra-IFT54 and the activated Dendra<sup>RED</sup> speckle. d-f) Tip turnaround of

activated 2xDendra-IFT54 speckles. g-i) Note movements of the 2xdendra-IFT54 speckle (arrow). Bars = 2  $\mu$ m, 2 s.

**C)** Distribution of the tip dwell time of activated 2xDendra-IFT54 speckles at the tip.

**D)** Kymograms showing the tip turnaround of BBS4-mNG expressed at low levels in the corresponding *bbs4-2* mutant. Note that BBS4-mNG after reaching the tip often undergoes slow diffusion migrating away from the tip (a, b, e) but particles remaining more stationary at the tip were observed as well (d). Open red arrowhead, arrival at the tip; closed arrowheads, onset of retrograde IFT. Note split of the BBS4-mNG particle depicted in b with one particle returning to the flagellar base by IFT and the other apparently by diffusion. Bars = 2  $\mu$ m, 2 s.

**E)** Distribution of the tip dwell time of BBS4-mNG at the tip.

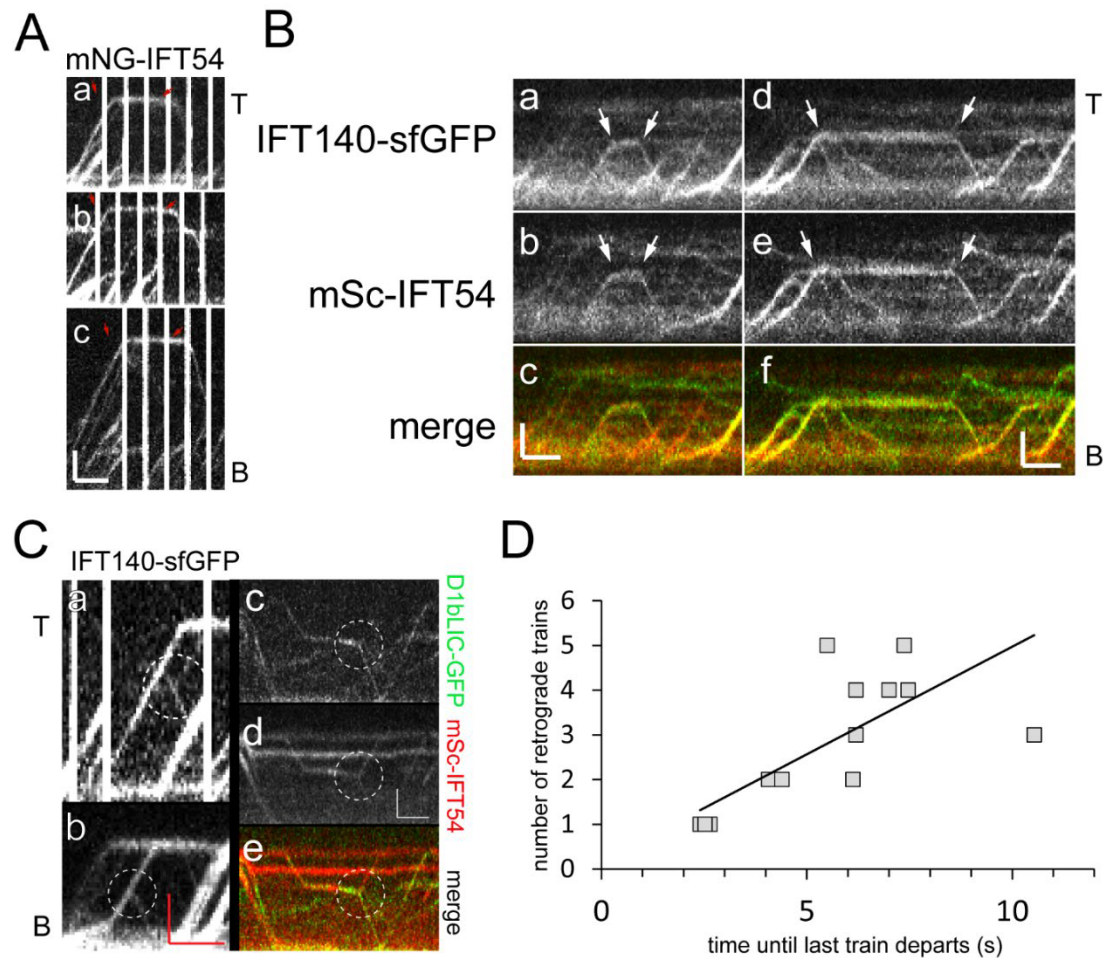


**Fig. S2. IFT velocity and dwell time are temperature-dependent**

**A)** Kymographs of *ift46-1* IFT46-YFP taken at 16, 21, and 27 °C. White brackets indicate the length of the pauses at the tip. Bars = 2 μm, 2 s.

**B)** The effect of temperature on the minimum tip dwell time of mSc-IFT54 and IFT40-sfGFP imaged at 16 and 27 °C and IFT46-YFP imaged at 16, 21, and 27 °C. Error bars indicate standard deviation and the star symbol (\*) indicates a significant difference of >0.0001 (2-tailed T-test).

**C, D)** The effect of temperature on anterograde (C) and retrograde (D) IFT velocities of mSc-IFT54, and IFT40-sfGFP imaged at 16 and 27 °C and IFT46-YFP imaged at 16, 21, and 27 °C. Error bars indicate standard deviation and the star symbol (\*) indicates a significant difference of >0.0001 (2-tailed T-test).



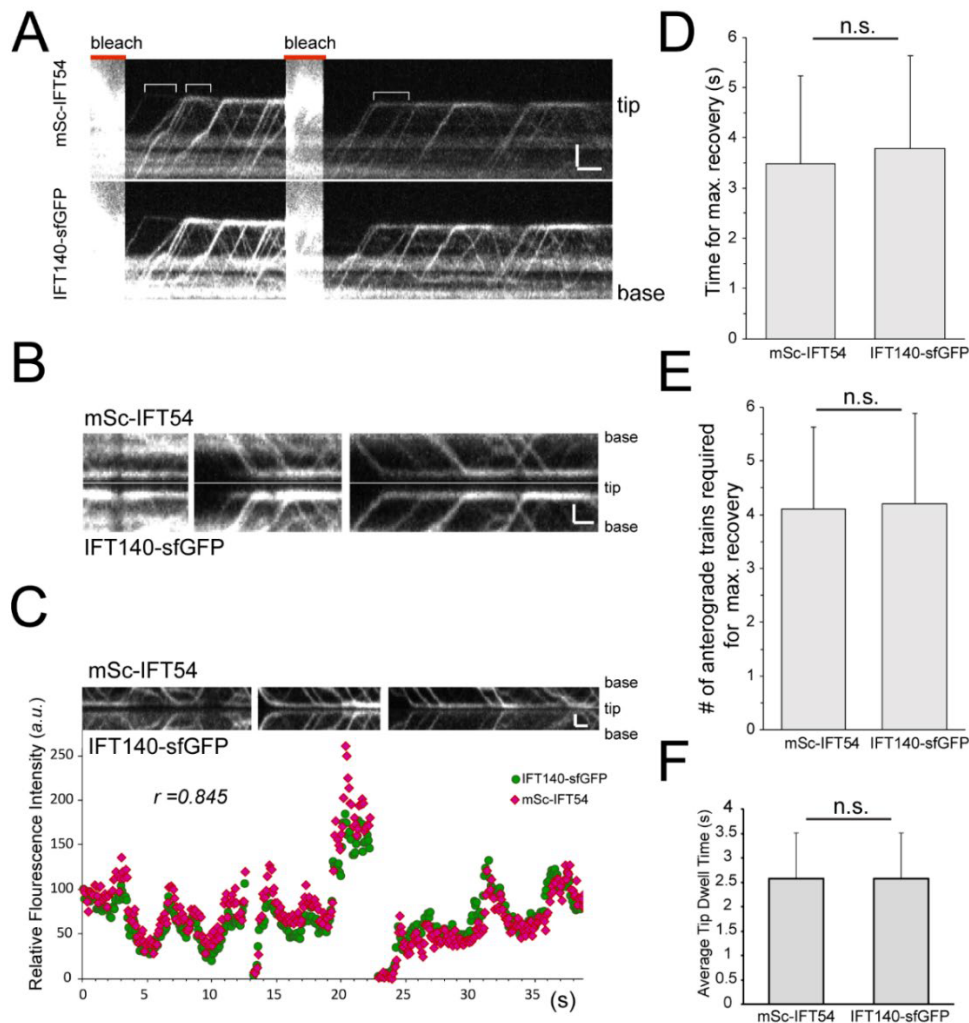
**Fig. S3.** Turnaround of unfragmented train at the tip and along the length of flagella

**A)** Kymograms showing turnaround of mNG-IFT54 trains without apparent fragmentation from photogate experiments. Bars = 2  $\mu$ m, 2 s.

**B)** Kymograms showing the turnaround of IFT140-sfGFP mSc-IFT54 trains along the length of flagella. Bars = 2  $\mu$ m, 2 s.

**C)** Kymograms showing train fragmentation along the length of flagella for the proteins indicated. Bars = 2  $\mu$ m, 2 s.

**D)** Plot of time between the arrival of a single anterograde train and the departure of the last derived retrograde train vs. the total number of retrograde trains produced. The data are based on mNG-IFT54 suggest that large anterograde trains as assessed by the number of resulting retrograde fragments need more time to convert.



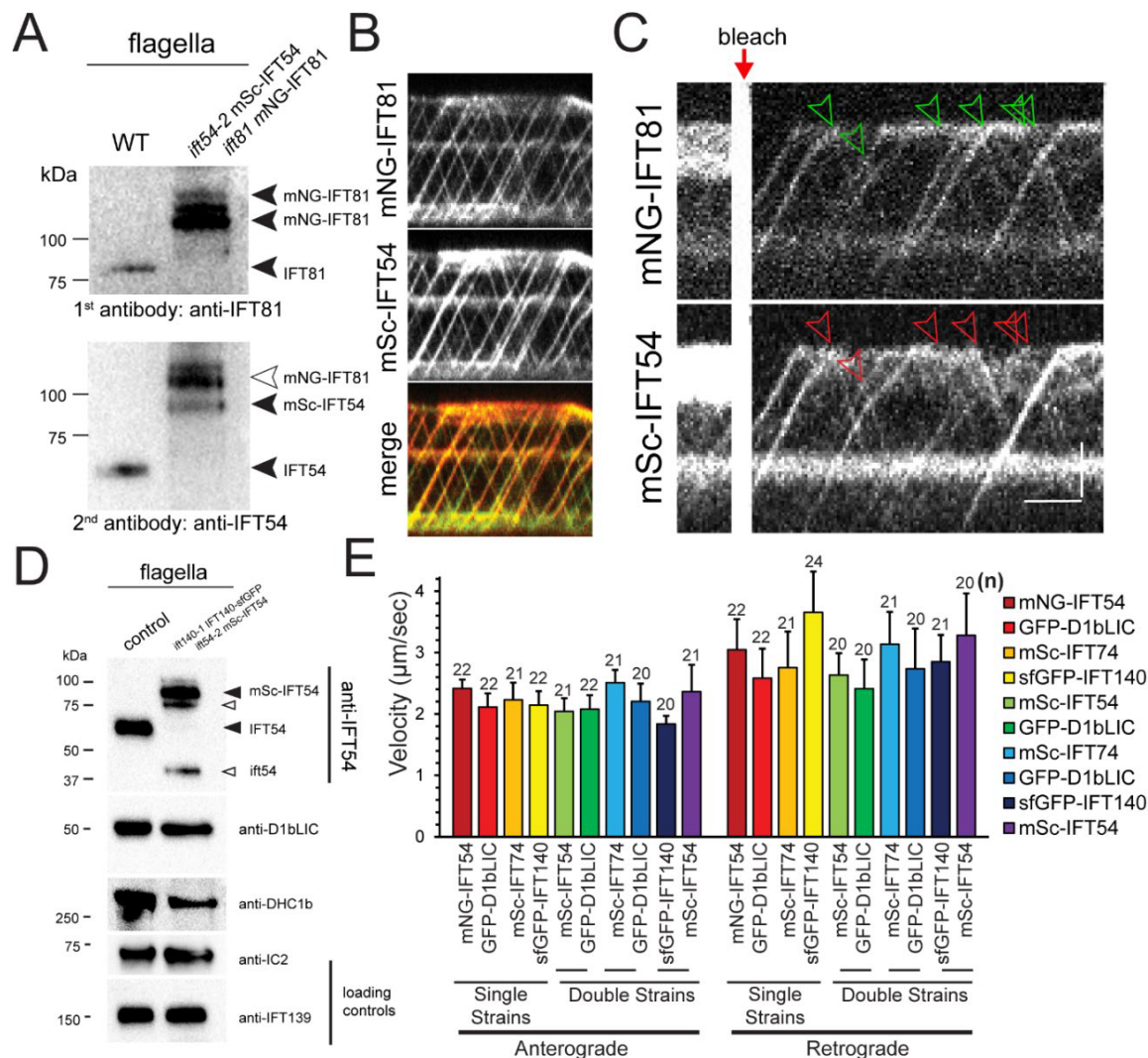
**Fig. S4.** The presence of IFT140-sfGFP and mSc-IFT54 at the flagellar tip is balanced

**A - C)** Kymograms of tip-FRAP experiments of tagged IFT140 and IFT54 expressed in the corresponding double mutant background. In B and C, the kymogram of mSc-IFT54 has been inverted to allow for a better comparison of the tip signals. In C, a quantitative analysis of the signal strengths at the tip and the Pearson coefficient were included. Bars = 2 μm, 2 s for A, and 1 μm, 1 s for B and C.

**D)** Time needed for full recovery of the tip signal of IFT140-sfGFP and mSc-IFT54 in tip-FRAP experiments.

**E)** Average number of anterograde trains arriving at the to reach full fluorescence recovery for IFT140-sfGFP and mSc-IFT54.

**F)** Average dwell time of IFT140-sfGFP and mSc-IFT54 at the flagellar tip.



**Fig. S5. Co-migration of mNG-IFT81 (IFT-B1) and mSc-IFT54 (IFT-B2) during anterograde and retrograde IFT**

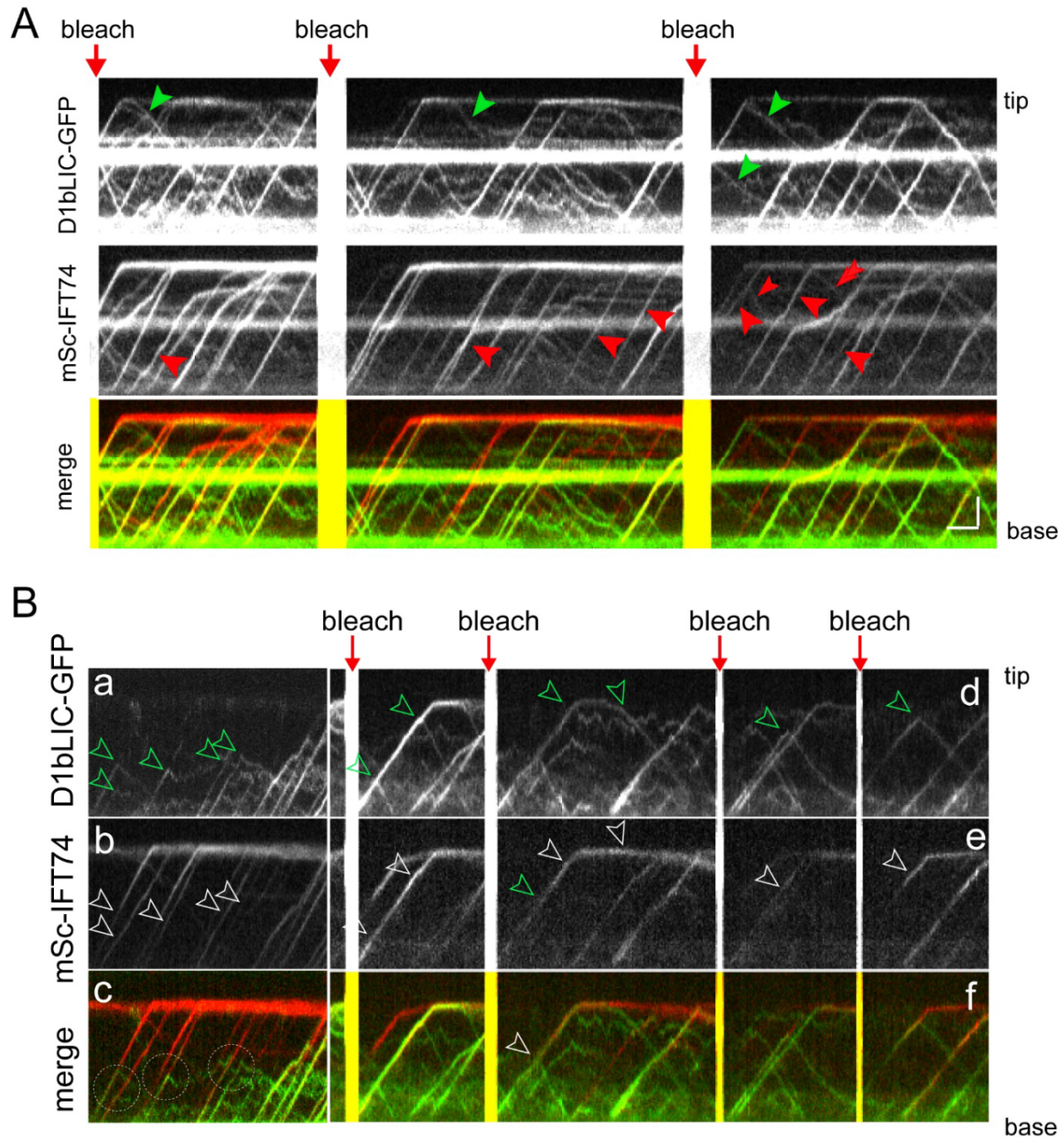
**A)** Western blot of flagella isolated from wild-type and *ift54-2* mSc-IFT54 *ift81* mNG-IFT81 cells. The membrane was first stained with anti-IFT81 and subsequently with anti-IFT54.

**B)** Kymographs showing IFT of mNG-IFT81 and mSc-IFT54. Scale bars=2μm, 2sec.

**C)** Tip-Frap experiment of the *ift54-2* mSc-IFT54 *ift81* mNG-IFT81 showing the presence of tagged IFT54 and IFT81 in retrograde trains (arrowheads).

**D)** Western blot of flagella isolated from a control and the *ift140-1* IFT140-sfGFP *ift54-2* mSC-IFT54 strain. According to the band intensities of the loading controls, the sample of the double mutant double rescue strain is slightly underloaded indicating a moderate reduction of IFT dynein in its flagella.

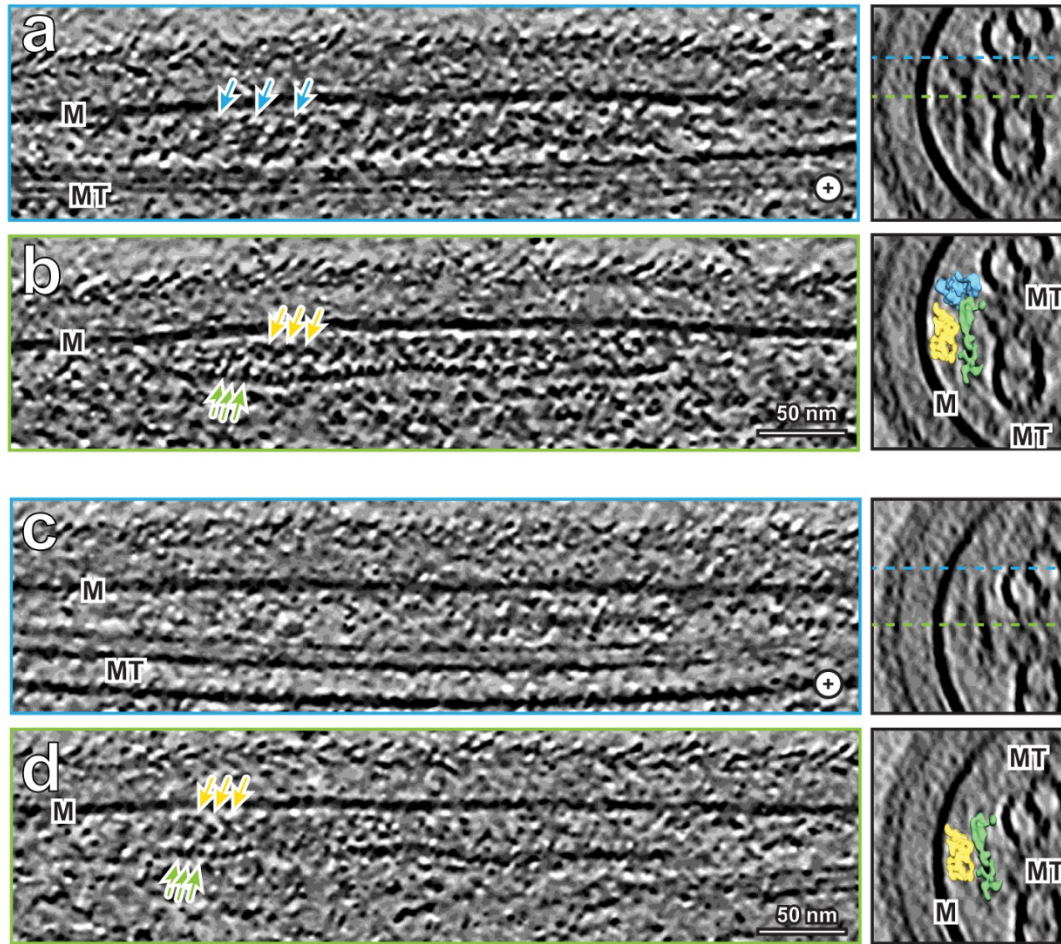
**E)** Anterograde and retrograde velocity of the tagged IFT proteins in selected single and double rescue strains.



**Fig. S6.** Separation of D1bLIC-GFP and mSc-IFT74 during IFT

A) Kymographs of a tip-FRAP experiments showing unbleached D1bLIC-GFP only trains first departing the tip (green arrowheads). Red arrowheads indicate trains predominately containing unbleached mSc-IFT74. Red double arrowhead marks an mSc-IFT74 only retrograde train indicating combination with bleached D1bLIC-GFP at the tip. Bar = 2  $\mu$ m and 2 s.

**B)** Gallery of kymograms from tip-FRAP experiments showing dissociation of D1bLIC-GFP from mSc-IFT74 trains. a-c) D1bLIC-GFP dissociating from anterograde trains moves by diffusion in the flagellar shaft. d-f) Separation of D1bLIC-GFP from anterograde mSc-IFT74 trains and early return by IFT or diffusion. Arrowheads indicate the points of separation. Bars = 2  $\mu$ m and 2 s.

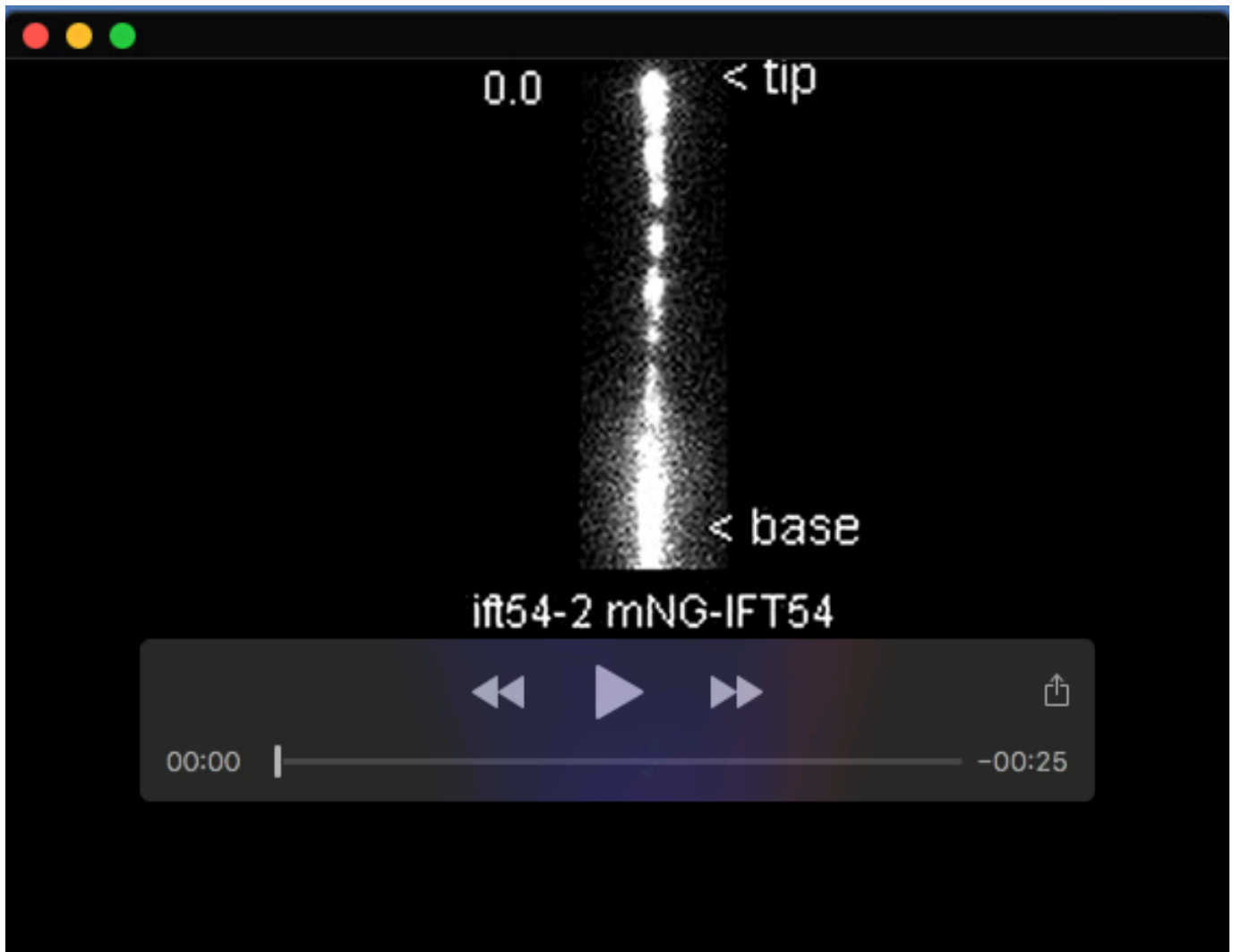


**Fig. S7.** *Absence of dynein from anterograde trains*

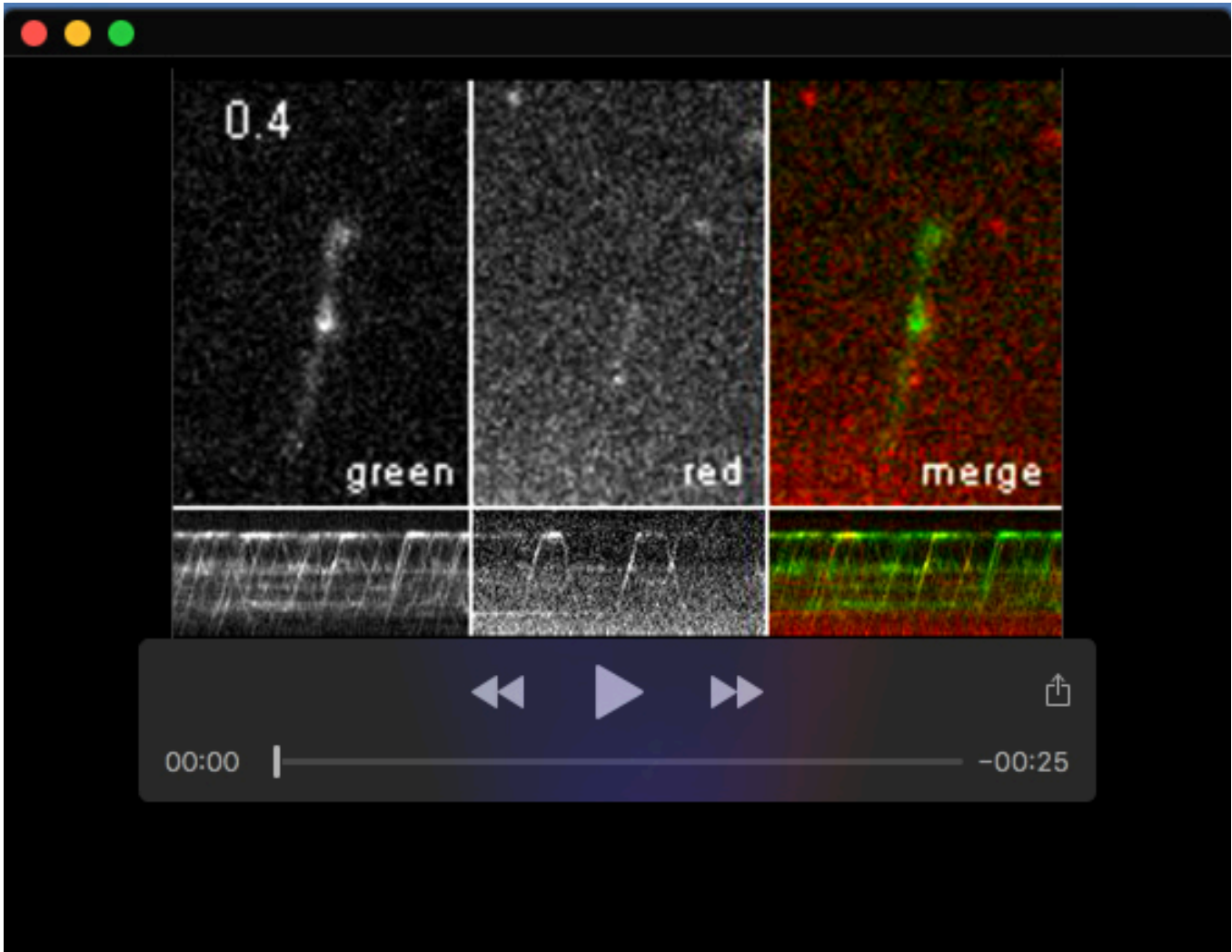
Slices of a tomogram from the mutant, showing an example of a train with dynein-1b (a and b) and without dynein-1b (c and d). The height of the slices is indicated in the cross sections on the right, green cutting through IFT-A and IFT-B and blue cutting through dynein-1b (if present). Some repeats of dynein-1b (blue), IFT-A (yellow) and IFT-B (green) are indicated with arrows. M: membrane, MT: microtubule, +: direction of the tip.

**Table S1.** *Chlamydomonas* strains used in this study.

Subcomplex(es)	Strain	Source
IFT-A	<i>ift140-1</i> IFT140-sfGFP (or IFT140-mC)	Picariello et al. (2019)
	<i>ift43</i> IFT43-YFP	Zhu et al. (2017)
IFT-B	<i>ift81-1</i> mNG-IFT81	this study
	<i>ift74-2</i> mSc-IFT74	this study
	<i>ift46</i> IFT46-YFP	Lv et al. (2017)
	<i>ift54-2</i> mNG-IFT54 (or mSc-IFT54 or 2xDendra-IFT54)	Wingfield et al. (2017) and this study
BBSome	<i>bbs4-1</i> BBS4-mNG	this study
Dynein	<i>d1blic</i> D1bLIC-GFP	Reck et al. (2016)
IFT-A/IFT-B	<i>ift140-1</i> IFT140-sfGFP <i>ift54-2</i> mSc-IFT54	this study
IFT-B1/IFT-B2	<i>ift81-1</i> mNG-IFT81 <i>ift54-2</i> mSc-IFT54	this study
IFT-B2/IFT-Dynein	<i>ift54-2</i> mSc-IFT54 <i>d1blic</i> D1bLIC-GFP	this study
	<i>ift74-2</i> mSc-IFT74 <i>d1blic</i> D1bLIC-GFP	this study

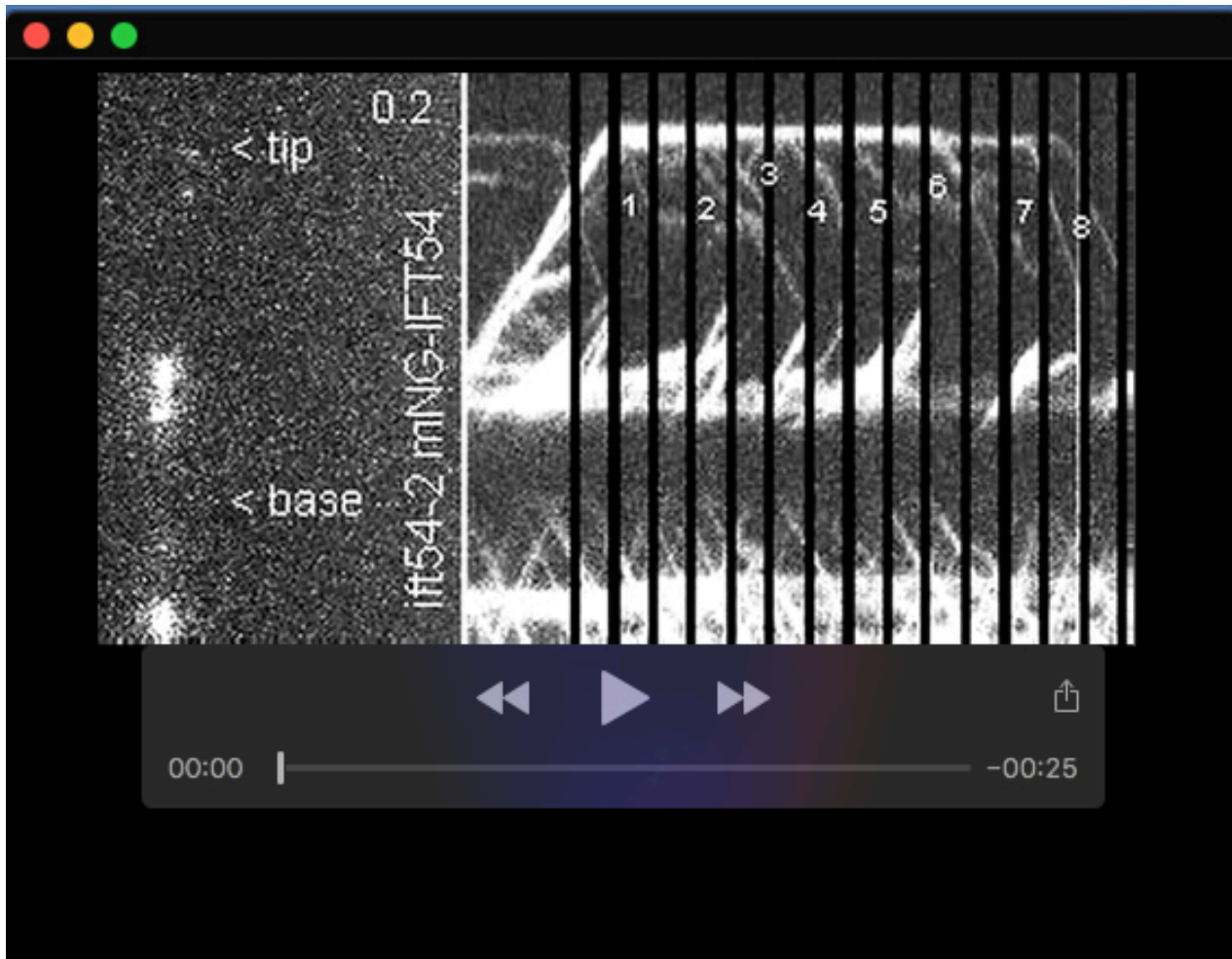


**Movie 1.** *mNG-IFT54 dwells at the flagellar tip* The distal portion of a flagellum of the *ift54-2* mNG-IFT54 was repeatedly bleached. Anterograde trains reenter the bleached area and after a pause retrograde traffic re-commences (indicated by exclamation marks). The movie was recorded at 10 fps and the timer displays seconds.



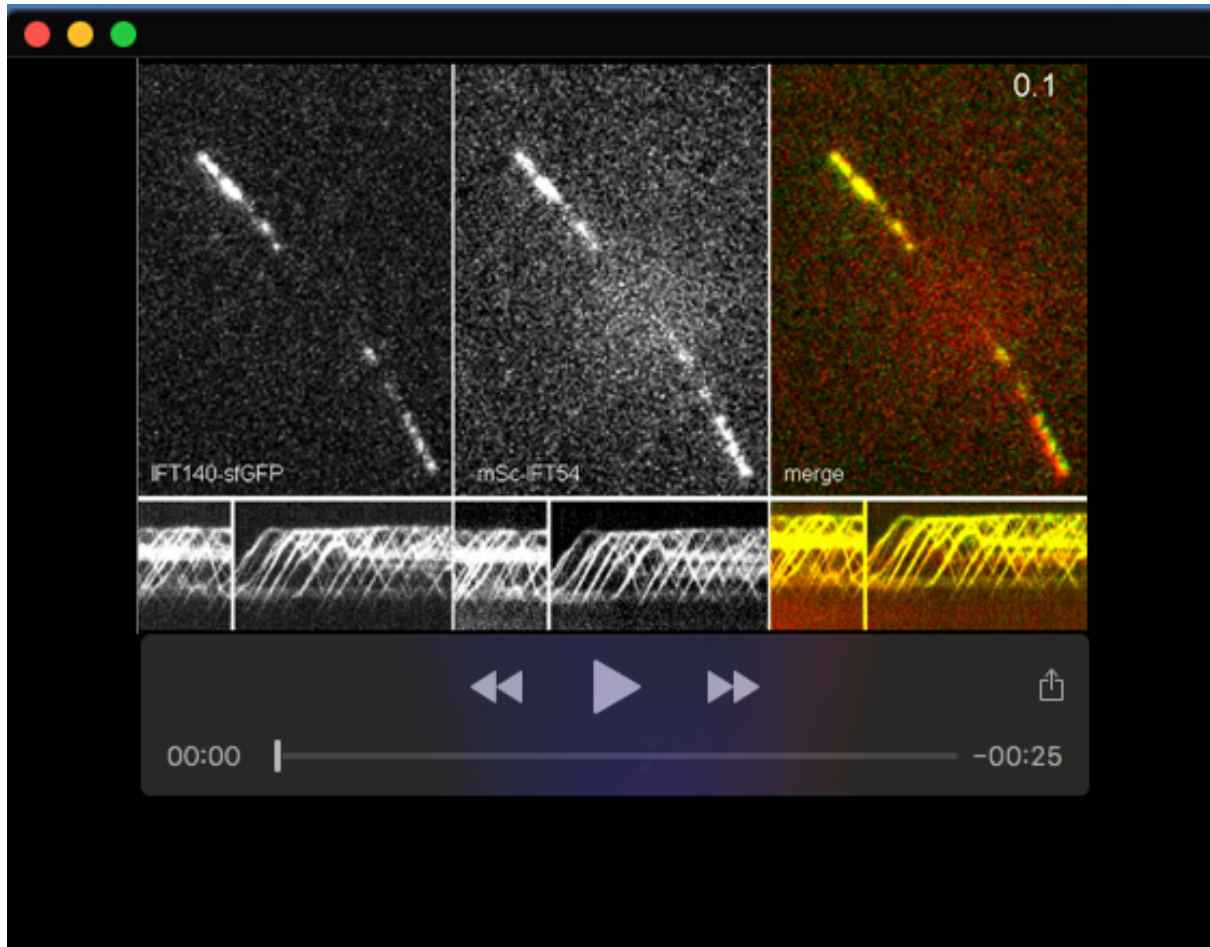
**Movie 2.** *2xDendra-IFT54 speckles pause at the tip*

In the ift54-2 2xDendra-IFT54 a subset of the fusion protein auto-converts from the green emitting into the red-emitting Dendra form allowing to visualize individual IFT54 speckles during turnaround at the tip. The star marks the time points when Dendra<sup>RED</sup>-IFT54 speckles approach the tip. The corresponding kymographs are shown at the bottom. The movie was recorded at 20fps and the timer displays seconds.



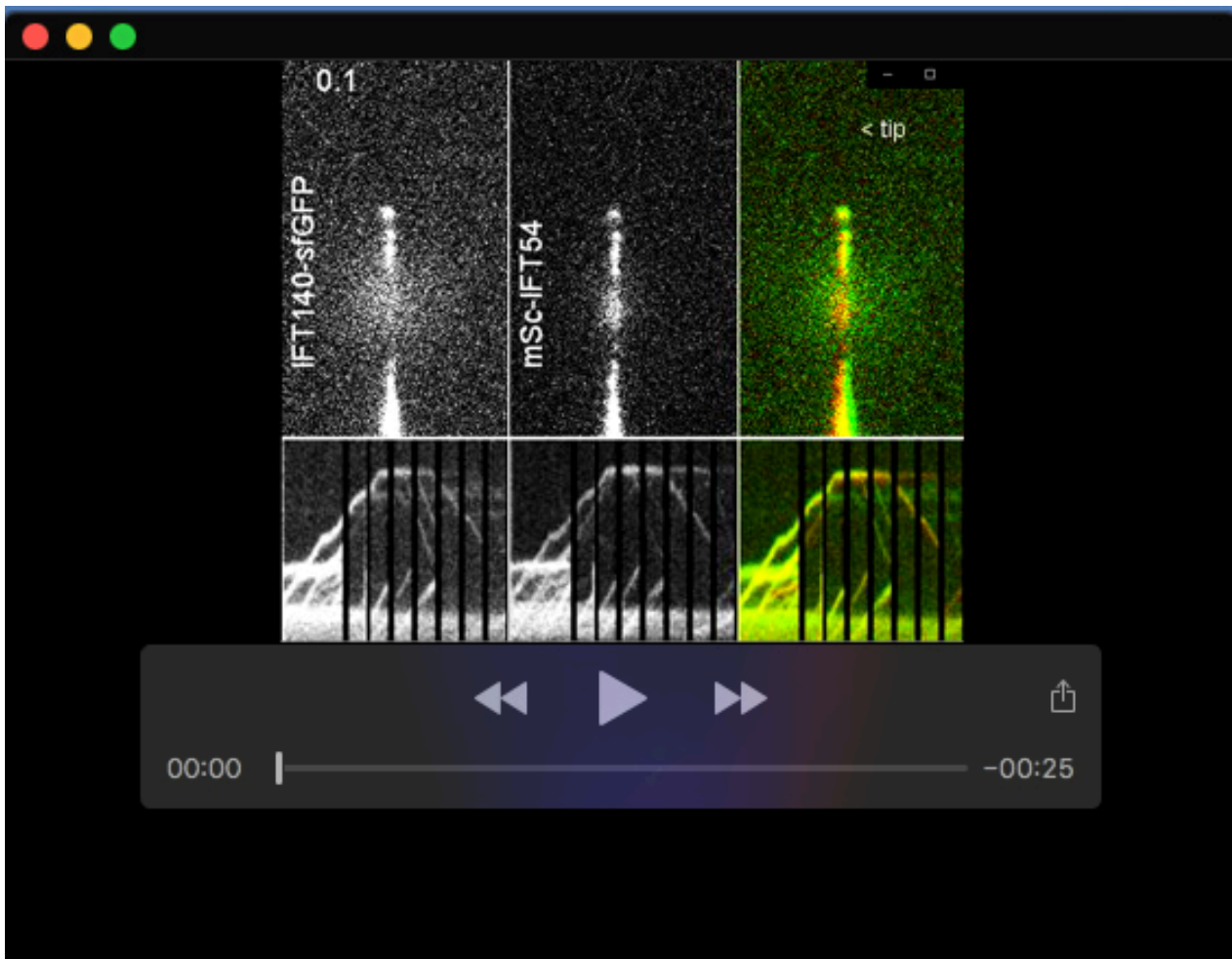
**Movie 3.** *Photogate showing fragmentation of an mNG-IFT54 train*

Single color photogate experiment showing a large anterograde mNG-IFT54 train fragmenting into eight retrograde trains. The overexposed frames were blackened and the corresponding kymogram is shown on the right. The movie was recorded at 10fps and the timer displays seconds.

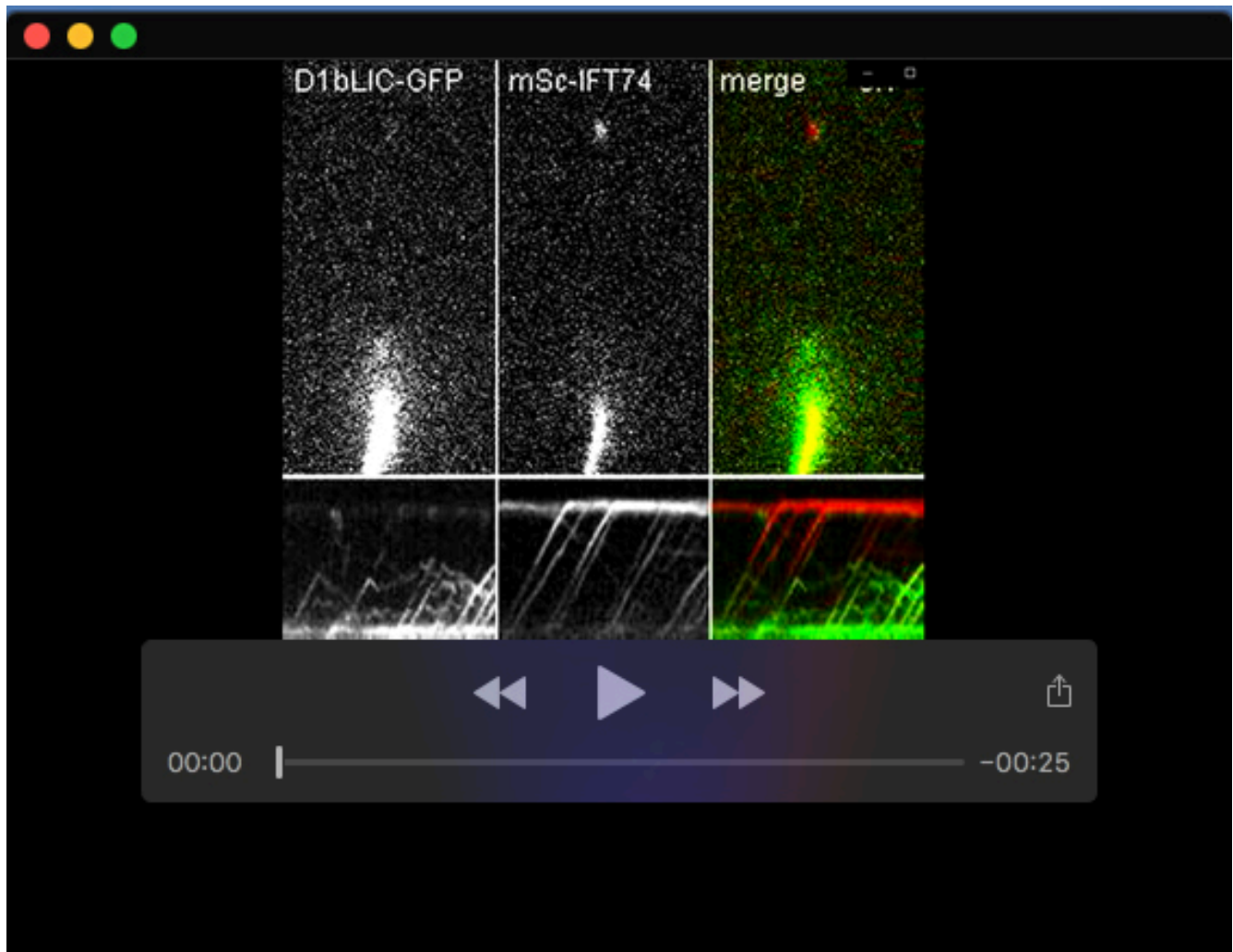


**Movie 4.** *Two-color tip-FRAP of IFT140-sfGFP and mSc-IFT54*

After bleaching the flagellar tip using a flash with a focused laser beam IFT140-sfGFP and mSc-IFT54 simultaneously enter the bleached area and are both present in the resulting retrograde trains. The corresponding kymograms are shown at the bottom. The movie was recorded at 10fps and the timer displays seconds.



**Movie 5.** *IFT140-sfGFP* and *mSc-IFT54* largely remain associated during tip turn around 2-color photogate experiment using the *ift140-1**IFT140-sfGFP ift54-2* *mSc-IFT54* double-mutant-double-rescue strain. The overexposed frames were blackened. The corresponding kymograms are shown at the bottom. The movie was recorded at 10fps and the timer displays seconds.



**Movie 6.** *D1bLIC-GFP dissociates from anterograde mSc-IFT74 trains*

Recording of the *d1bLIC* D1bLIC-GFP *ift74-2* mSc-IFT74 strain showing the absence and detachment of D1bLIC-GFP from mSc-IFT74 trains. The corresponding kymograms are shown at the bottom. The movie was recorded at 10fps and the timer displays seconds.