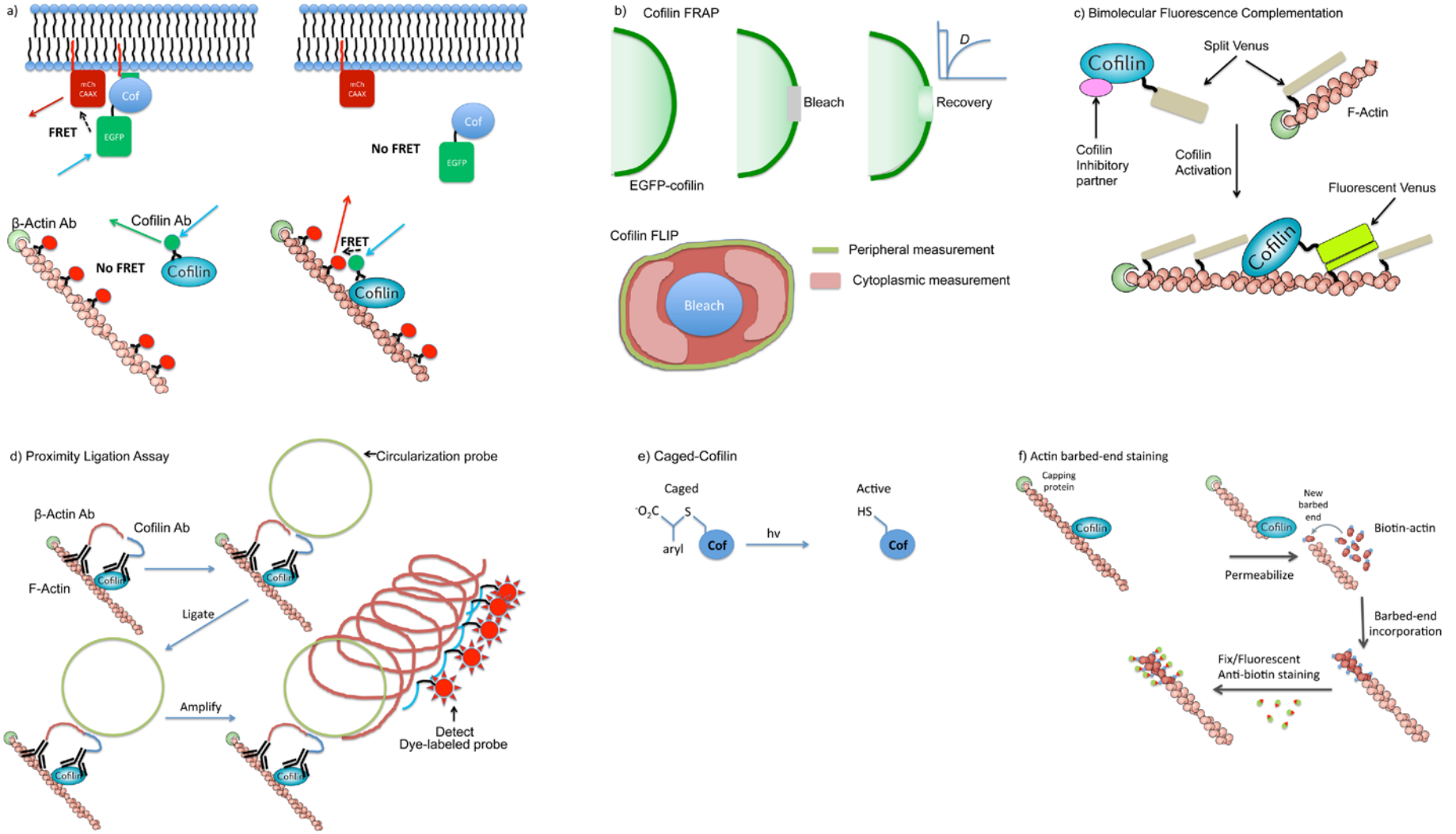
In format provided by Condeelis *et al.* (JULY 2013)



Supplementary information S1 (box 1) | **High-resolution methods to study cofilin in migrating cells**

Fluorescence resonance energy transfer (FRET)

The dynamics of the dissociation of active cofilin from the plasma membrane (see the figure, part **a**) can be monitored by FRET between GFP–cofilin and mCherry-CAAX[1] in living eukaryotic cells. The use of expressed GFP-tagged cofilin in eukaryotic cells has been validated: GFP-cofilin interacts with actin in cell extracts (using co-immunoprecipitation); it severs actin filaments *in vitro* (as shown by co-sedimentation assays); and has been observed to fragment individual actin filaments in an *in vitro* assay (using total internal reflection (TIRF) microscopy)[2]. However, in budding yeast[3] and fission yeast[4], the GFP–tagged cofilin fusion protein is not fully functional and had to be overexpressed to rescue cofilin deletion phenotypes.The binding of endogenous cofilin to F-actin can also be measured by antibody-based FRET (for example, between immunolabelled endogenous cofilin and F-actin[1,5,6] in fixed cells). These two FRET signals, GFP and/or mCherry and antibody-based FRET, can be studied independently during cell protrusion even though they are within the diffraction limited spot of light microscopes. Both of these independent methods yield similar results in eukaryotic cells[1,6].

Fluorescence loss in photobleaching (FLIP)

The mobility of plasma membrane- and F-actin-bound cofilin can be measured by bleaching cytosolic GFP–cofilin (see the figure, part **b**). Using appropriate conditions, mobility differences between the plasma membrane- and F-actin-bound fractions are quantified[1,7]. Fluorescence recovery after photobleaching (FRAP) experiments can be used to analyse the lateral mobility of plasma membrane-bound cofilin.

Bimolecular fluorescence complementation (BiFC)

Binding of cofilin to F-actin and G-actin can be monitored using Venus fluorescent protein (a derivative of YFP) that is split between actin and cofilin (see the figure, part **c**). It does not distinguish between F-actin- and G-actin-bound cofilin. The actin–cofilin binding induced by this approach is irreversible[8].

Proximity ligation assay (PLA)

This method can be used to study F-actin and G-actin binding to endogenous cofilin at single-molecule resolution[5] (see the figure, part **d**). PLA uses antibodies conjugated to oligonucleotides that form complementary segments to a circularization probe, which are *in situ* ligated and circularly amplified. The amplified DNA can be detected by dye-labelled probe complementation. PLA has an extremely high signal-to-noise ratio for the detection of protein–protein binding *in situ*[9].

Caged cofilin

A constitutively active S3A cofilin phosphorylation mimetic mutant (in which Ser3 was replaced with Asp) is chemically caged using a photolabile moiety (see the figure, part **e**). Upon photocleavage, activated cofilin is released. This technique can be used to initiate focal activation of cofilin in live cells and has been applied to validate the new model for the activation of cofilin at the leading edge of locomotory protrusions. The system is not reversible, and therefore the potential effects on cells from the accumulation of active cofilin must be considered[10].

Cofilin-dependent barbed ends *in situ*

By labelling actin barbed ends with fluorescently tagged G-actin, cofilin severing and/or barbed end generation activity can be measured *in situ*[6,11] (see the figure, part **f**).

1. van Rheenen, J. *et al.* EGF-induced PIP2 hydrolysis releases and activates cofilin locally in carcinoma cells. *J. Cell Biol.* **179**, 1247–1259 (2007).
2. Lai, F. P. *et al.* Arp2/3 complex interactions and actin network turnover in lamellipodia. *EMBO J.* **27**, 982–992 (2008).
3. Okreglak, V. & Drubin, D. G. Cofilin recruitment and function during actin-mediated endocytosis dictated by actin nucleotide state. *J. Cell Biol.* **178**, 1251–1264 (2007).
4. Chen, Q. & Pollard, T. D. Actin filament severing by cofilin is more important for assembly than constriction of the cytokinetic contractile ring. *J. Cell Biol.* **195**, 485–498 (2011).
5. Bravo-Cordero, J. J. *et al.* Spatial regulation of RhoC activity defines protrusion formation in migrating cells. *J. Cell Sci.* 23 May 2013 (doi:10.1242/jcs.123547).
6. Oser, M. *et al.* Cortactin regulates cofilin and N-WASp activities to control the stages of invadopodium assembly and maturation. *J. Cell Biol.* **186**, 571–587 (2009).
7. Leyman, S. *et al.* Unbalancing the phosphatidylinositol-4,5-bisphosphate–cofilin interaction impairs cell steering. *Mol. Biol. Cell* **20**, 4509–4523 (2009).
8. Ohashi, K., Kiuchi, T., Shoji, K., Sampei, K. & Mizuno, K. Visualization of cofilin-actin and Ras–Raf interactions by bimolecular fluorescence complementation assays using a new pair of split Venus fragments. *Biotechniques* **52**, 45–50 (2012).
9. Soderberg, O. *et al.* Direct observation of individual endogenous protein complexes *in situ* by proximity ligation. *Nature Methods* **3**, 995–1000 (2006).
10. Ghosh, M. *et al.* Cofilin promotes actin polymerization and defines the direction of cell motility. *Science* **304**, 743–746 (2004).
11. Mouneimne, G. *et al.* Phospholipase C and cofilin are required for carcinoma cell directionality in response to EGF stimulation. *J. Cell Biol.* **166**, 697–708 (2004).