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High-Resolution Live-Cell Imaging and Time-Lapse Microscopy of Invadopodium Dynamics and Tracking Analysis

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Abstract

Invadopodia are specialized structures of cancer cells which aid in cancer cell invasion and metastasis. Therefore, studying the early steps of invadopodium assembly and its life cycle at the subcellular level by using high spatiotemporal resolution imaging provides an opportunity for understanding the signaling mechanisms involved in this very important process. In this chapter, we describe the design of a custom-built high-resolution fluorescence microscope which makes this challenging imaging possible. We also describe an ImageJ plugin that we have developed for tracking of invadopodia and lifetime analysis.

Keywords

Live-cell imaging; Time-lapse microscopy; Autofocus; Invadopodia; Breast carcinoma cells; Gelatin degradation; Particle tracking

1 Introduction

Metastasis is the leading cause of death in most cancer patients, as opposed to the growth of the primary tumor, which can be managed clinically to a large extent by surgery and chemotherapy. Invadopodia are specialized protrusive structures found in cancer cells, with a diameter around 0.5–1 μm and length of a couple of microns [1]. These structures are proteolytically active and degrade extracellular matrix (ECM) which aids in cancer cell invasion into the surrounding tissue and blood vessels leading to metastasis [2–4]. Since their first discovery in src-transformed fibroblasts [5], invadopodia have been found in many types of cancer cells including: breast [3, 6–8], melanoma [9, 10], head and neck [11], glioma [12], colon [13], pancreatic [14], and prostate [15].

By utilizing a 2D model in which cancer cells are plated on ECM coated surfaces, the mechanism of invadopodium formation and regulation has been studied in great detail. Although many invadopodium-associated proteins (e.g., cortactin, N-WASp, Tks5, Cofilin, Nck1, p190RhoGAP [7, 16–18]) have been identified, the molecular interaction(s) during invadopodium initiation is not well understood. We were interested in imaging various invadopodium proteins during invadopodium precursor assembly at high spatial and temporal resolution (on the order of seconds). To achieve these challenging imaging requirements, we have developed a robust custom-built wide-field fluorescence microscope. In this chapter, we provide technical details on the microscope design and data acquisition. To analyze the arrival of different invadopodium proteins, invadopodia lifetimes and trajectories, we have also developed an ImageJ plugin called “Invadopodia tracker.” This plugin tracks multiple invadopodia in a cell throughout the whole time-lapse sequence. A step-by-step invadopodia tracking analysis procedure is also described.

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2 Materials

2.1 Microscope Design

1. Excitation, emission, and ND filters (*see* Table 1).
2. Mercury arc lamp and controller (Chiu Technical Corporation, Mercury-100 W).
3. Dual filter wheel with six positions each for Excitation and ND filters (Ludl Electronics, Dual Filter Wheel).
4. 8-position filter wheel for emission filters (Applied Scientific Instrumentation, FW1000).
5. Continuous Reflective-Interface Feedback Focus unit (Applied Scientific Instrumentation, CRIFF).
6. Z-focus Stepper Motor (Applied Scientific Instrumentation, MFC2000Z Axis Drive).
7. Fast piezo controlled Z-focus drive (Physik Instrumente, PIFoc).
8. Linear encoded XY Stage (Applied Scientific Instrumentation, MFC2000).
9. Glass reflector, 6 % reflection/94 % transmission (Chroma, 6/94bs).
10. 60× 1.42 NA oil, Plan Apo N objective (Olympus, part# 1-U2B933).
11. Two channel image splitter (Optical Insights, Dual-View).
12. High Performance 12 bit CCD camera (Cooke, SensiCamQE).

2.2 Labeling Gelatin with Alexa 405 Dye

1. Bio-Gel P-30 Gel (Bio-Rad cat#150-4154).
2. PBS with 2 mM sodium azide: Add 65 mg sodium azide in 500 mL PBS. Use this PBS for all the steps described in Subheadings 2.2 and 3.2.
3. Glass column (Bio-Rad, cat# 737-1021).
4. Alexa Fluor 405 dye (Life Technologies, cat#A30000).
5. 0.2 % gelatin solution: Add 50 mg gelatin from porcine skin (Sigma-Aldrich, cat# G2500) into 25 mL PBS. Vortex briefly to mix and leave in the 37 °C water bath for ½–1 h. During the incubation period, take the tube out two times and vortex briefly.
6. 1 M sodium bicarbonate solution: 840.1 mg sodium bicarbonate powder in 10 mL ddH_2O; prepare fresh every time.
7. DMSO.
8. UV lamp.

2.3 Preparation of Gelatin-Coated MatTek Dishes

1. MatTek dish (MatTek corporation, cat# P35G-1.5-14-C).
2. 1 N HCl.
3. 2 mg/mL Poly-L-lysine solution: Add 12.5 mL ddH_2O to 25 mg poly-L-lysine powder (Sigma-Aldrich, cat# P1399). The solution can be stored at 4 °C for up to 1 year.

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4. Prepare 0.2 % gelatin solution as described in Subheading 2.2, **item 5**.
5. 0.2 % glutaraldehyde solution: Add 160 μL of 25 % glutaraldehyde stock (Sigma-Aldrich, cat#G5882) into 20 mL PBS, leave on ice.
6. Sodium borohydride (Sigma-Aldrich, cat#452882).
7. 70 % ethanol solution.
8. 10× penicillin/streptomycin solution: Add 2 mL, 100× penicillin/treptomycin stock (Life Technologies, cat# 15140-122) into 18 mL PBS.

2.4 Transfection of MTLn3 Cells and Preparing Cells for Live Imaging

1. MTLn3 cells [19].
2. Culture medium: MEM Alpha (Life Technologies, cat#12561-056), 5 % fetal bovine serum (FBS) (Gemini Bio-products, cat#100106) and 1× penicillin/streptomycin.
3. 0.05 % trypsin–EDTA (Life Technologies).
4. Lipofectamine 2000 (Life Technologies).
5. Opti-MEM medium (Life Technologies).
6. DNA constructs (e.g., TagRFP-cortactin and GFP-Cofilin [17, 20]).
7. Serum starvation medium: Dissolve 0.345 g bovine serum albumin (BSA; Fisher Scientific, cat# BP1600-100) in 5 mL ddH_2O. Syringe filter through a 0.2 μm size filter. Add 2.5 mL of this solution into 47.5 mL L-15 medium (Life Technologies, cat# 21083). Place at 37 °C before use.
8. Steady state imaging medium: L-15 + 5 % FBS.

2.5 Live Imaging of Invadopodia

1. 10 nM EGF: Dilute 5 μL EGF stock (50 μM, Life Technologies, cat# 53003-018) into 245 μL serum starvation medium. Add 25 μL of this solution into 2 mL serum starvation medium.

3 Methods

3.1 Microscope Design

The microscope system (Fig. 1a) is built upon an Olympus IX-70 microscope stand with the following custom modifications:

1. The use of a glass reflector in place of a dichroic mirror in the filter turret of the microscope enables complete flexibility in the choice of fluorophores and efficient collection of emitted photons (Fig. 1b). However, as only 6 % of the excitation light makes it to the sample, a bright light source is required. For this purpose we have chosen a mercury arc lamp over other options (e.g., xenon or LED source). In cases where the fluorophores chosen were not bright enough for use with the glass reflector, other filter turret positions were loaded with multi-band dichroics specific for particular applications (e.g., CFP-YFP-RFP, GFP-RFP and Cy5 imaging).
2. Control of the excitation intensity is accomplished with ND filters in one wheel of a dual filter wheel system. The second wheel of this system is used to house excitation filters which allow separate and individual control of excitation wavelengths.

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3. Emission wavelengths were individually controlled with a separate filter wheel mounted in front of the CCD camera. The sensitivity of the measurements to drifts in focus necessitates an active autofocus module. For this purpose, a CRIFF unit utilizing a stepper motor z-focus drive was employed. To accommodate both the emission filter wheel and the CRIFF unit in the emission path of the microscope, a custom mount was designed and machined (Fig. 1c, d, *see* Note 1).
4. Additional fast z-scanning capabilities are attained by use of a piezo controlled focus mount. Mosaicing and multiple sample position acquisition are facilitated by a motorized xy stage equipped with linear encoders for accurate stage repositioning.
5. In some experiments, performing sequential imaging would be too slow to capture the rapid dynamics of the biological process of interest. In these cases, the emission filter wheel is put into an "open" position and an image splitter device (Dual-View) is placed in the emission path. This device splits the image into two halves, passing one through a long pass filter and the other through a short pass filter, and then projects the two images onto each half of the CCD chip.
6. The microscope utilizes a high numerical aperture objective lens (60×, 1.42 NA) for high resolution imaging.

For a list of fluorophores (and dyes) which can be imaged sequentially or simultaneously on the microscope, *see* Table 2.

3.2 Labeling Gelatin with Alexa 405 Dye

1. A day before labeling the gelatin, take 2 g Bio-Gel P-30 powder and add 36 mL PBS, mix by gently inverting the tube 5–6 times and leave at room temperature (RT). Hold the glass column vertical by clamping on a stand. Mix Bio-Gel slurry from the previous day by pipetting up and down and transfer it to the column until it is full. The column will start draining PBS from the bottom. As the top level of packed column drops, add mixed Bio-Gel slurry to the column. Leave approximately a 3 cm space with PBS above the packed column. The column can be stored at 4 °C for up to 1 week (*see* Notes 2 and [3]).
2. While the column is draining in **step 1** above, bring 0.2% gelatin solution to RT. Transfer 2 mL gelatin solution into a 15 mL conical tube. Add 200 μL 1 M sodium bicarbonate solution.
3. Briefly centrifuge Alexa 405 dye containing tube to dislodge any dye stuck in the cap. Add 100 μL DMSO to 1 mg Alexa 405 dye, mix by pipetting up and down 3–4 times. Add the dye/DMSO solution into 2.2 mL gelatin solution from **step 2**. Cover the conical tube with aluminum foil and rotate at RT for 1 h.
4. After the column in **step 1** has stopped draining, transfer the dye–gelatin solution from **step 3** to the top of the column. After all the dye–gelatin solution enters the column, add PBS gently to the top.
5. After 15 min, check the dye–gelatin solution in the column with a UV lamp. It should look like a bright blue smear through the column. As the dye–gelatin

[1]The use of CRIFF unit requires a high numerical aperture objective e.g., 60×, 1.42 NA, because it works on the total internal reflection (TIR) principle.

[2]While packing the column make sure that the top of the packed column does not dry up. As soon as the PBS level falls off, replenish the top with more PBS.

[3]If the column is packed properly then it will flow approximately 2–3 drops per min. If not, then transfer the column packing into a tube, add some PBS, mix, and transfer it to a thoroughly cleaned column.

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solution reaches the bottom of the column, start collecting the dye labeled gelatin solution into eppendorf tubes. Collect about 2–2.5 mL (*see* Note 4). This solution can be stored at 4 °C for up to 2 months.

3.3 Preparation of Gelatin-Coated MatTek Dishes (For Ten Dishes)

1. Add 300 μL 1 N HCl into the well of each MatTek dish. Incubate for 10 min at RT. Wash three times with PBS in 5 min intervals.
2. Dilute poly-L-lysine solution to 50 μg/mL (75 μL stock solution in 3 mL PBS). Put 300 μL into each well and incubate for 20 min at RT. Wash three times with PBS in 5 min intervals.
3. Take out 50 μL of Alexa 405 dye-labeled gelatin (prepared above in Subheading 3.2) into an eppendorf tube, cover it with aluminum foil and leave it on the bench for it to reach RT.
4. Once gelatin is completely dissolved, take it out of the water bath and leave it at RT to cool. Add 50 μL of Alexa 405 dye-labeled gelatin from **step 3** into 2 mL 0.2 % gelatin solution (final concentration 1:40). Mix by pipetting 3–4 times and leave the mixture in a 37 °C water bath for 5–10 min. Take the tube out and let it cool at RT for 5 min before placing 200 μL of the solution into each well of the MatTek dish. Leave for 10 min at RT (*see* Note 5).
5. For making unlabeled gelatin dishes, skip **steps 3** and **4** above. Place 200 μL of 0.2 % RT gelatin solution into each well of the MatTek dish and leave for 10 min at RT.
6. Wash three times with PBS in 5 min intervals. Transfer dishes on a tray filled with ice. Add 2 mL cold 0.2 % glutaraldehyde solution into each dish and incubate on ice for 15 min. Remove the dishes from ice and wash three times with PBS in 5 min intervals.
7. Prepare 5 mg/mL sodium borohydride solution (100 mg powder in 20 mL PBS) and immediately add 2 mL of this solution into each dish. Incubate for 15 min at RT. Wash three times with PBS in 5 min intervals.
8. Add 2 mL 70 % ethanol into each dish and incubate at RT for 15 min. Wash three times with PBS in 5 min intervals.
9. Add 2 mL 10× penicillin/streptomycin solution into each dish. Store dishes at 4 °C for up to 10 days.

3.4 Transfection of MTLn3 Cells and Preparing Cells for Live Imaging

1. The day before the transfection plate MTLn3 cells in 35 mm dishes at 1.2×10^5 cells/dish in 2 mL culture medium.
2. For each dish take two eppendorf tubes. Add 250 μL Opti-MEM into each tube. In the first tube add 0.5–1 μg of DNA and mix. In the second tube, add 4 μL of Lipofectamine 2000 and mix. Leave both tubes in the culture hood for 5 min.

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[4]The Alexa 405 dye–gelatin solution runs as a bright blue smear in the column. As the leading front reaches the bottom of the column, a slightly gray zone separating the Alexa 405 dye labeled gelatin (fast running fraction) with the Alexa 405 dye alone (slower running fraction) will become visible.

[5]If you start seeing many aggregated bright Alexa 405 dye particles in your Alexa 405 labeled gelatin MatTek dishes (which might interfere with imaging and affect degradation area calculation), then the pre-warmed dye–gelatin solution in Subheading 3.3 **step 4** can be centrifuged at 10,000 × *g* for 5 min before adding it to the wells of the MatTek dish.

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3. Mix DNA and Lipofectamine 2000 solutions together and leave in the hood for 20 min.
4. Wash MTLn3 cells with pre-warmed Opti-MEM solution two times and leave 500 μL Opti-MEM. Add 500 μL DNA–Lipofectamine mixture from **step 3** into each dish. Transfer cells into the cell culture incubator for 45 min.
5. Wash cells two times with cell culture medium and leave 2 mL culture media on cells. Transfer cells to the cell culture incubator and let the gene(s) of interest express for 6–8 h.
6. Wash gelatin coated MatTek dishes two times with the culture medium and leave 1 mL culture medium each dish. Transfer dishes in the incubator for at least 30 min. Trypsinize transfected cells and plate cells from each 35 mm dish into two gelatin coated MatTek dishes. Leave cells in the incubator overnight.

3.5 Live Imaging of Invadopodia

1. For EGF stimulation experiments: Wash cells two times with serum starvation medium, leave 2 mL serum starvation medium on cells. Incubate cells for 3–4 h in a 37 °C incubator (no CO_2). Transfer cells from the incubator to the microscope stage and take the cover off the MatTek dish. Depending on the number of fluorophores you want to image, set the imaging parameters. For two fluorophores (e.g., GFP and RFP), we typically image every 3 s for up to 5 min (Fig. 2a, b). After capturing 1–2 frames in each channel, pause imaging, add 2 mL 10 nM EGF (final concentration 5 nM) in the dish and resume imaging.
2. For steady state imaging: Wash cells two times with steady state imaging medium, leave 2 mL steady state imaging medium on cells. Transfer cells to the microscope stage, start imaging.
3. For both EGF stimulation and steady state imaging, using the autofocus unit helps in maintaining focus throughout the imaging period (Fig. 2c, d). To use the autofocus unit, before starting imaging, turn the CRIFF laser ON and calibrate the focus by following the vendor's protocol.

3.6 Invadopodia Tracking with "Invadopodia Tracker" Plugin

1. Put the plugin file Invadopodia_tracker.java (available upon request) under the plugins folder in ImageJ. Run "Plugins - > Compile and Run…," select Invadopodia_tracker.java file from the plugins folder. Ignore any error(s). Check to see that there is a new file named "Invadopodia_tracker.class" in the plugins folder. Restart ImageJ, "Invadopodia tracker" command should appear under Plugins menu in ImageJ.
2. Open the 16-bit time-lapse stack of a single fluorescence channel (Fig. 3a).
3. Go through the whole stack and choose the frame where invadopodia are clearly visible (typically after 1–2 min in EGF stimulation experiments). Using multi-point tool, select invadopodia- of-interest (Fig. 3b). Alternatively, invadopodia-of-interest can be automatically selected by "Process - > Find Maxima…" command and adjusting the noise tolerance.
4. Run "Invadopodia tracker" from the Plugins menu. A GUI will open asking for maximum invadopodium displacement from one frame to the next (a typical value is 3–5 pixels) and estimates of minimum and maximum number of particles considered to be invadopodia in the whole field per frame (Fig. 3c). A typical range for minimum and maximum number of invadopodia is 25–50 and 100–300,

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respectively. The user needs to optimize these numbers for correct invadopodia tracking (*see* Notes 6 and 7). For the next three parameters—Noise tolerance, Delta noise tolerance and maximum iterations, the user should start with default values—500, 20, 200 respectively (*see* Note 8).

5. After tracking is over, tracked invadopodia are marked in red circles and added as overlays (Fig. 3d, f; *see* Note 9). A log file, containing invadopodia centroids in each frame, is also generated (Fig. 3e).
6. Copy and paste invadopodia centroids into Excel. It will show up as three columns —first column is the frame number, second and third columns are the *x* and *y* coordinates of invadopodium centroid (Fig. 3g).
7. From the centroid data, invadopodium trajectories can be easily plotted in Excel (Fig. 3h).

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[6] In the case that "Invadopodia tracker" plugin does track invadopodia through only some frames but misses others, the sensitivity of the invadopodia detection can be enhanced by increasing the values for the estimates of minimum and maximum number of invadopodia. Conversely, if the tracker identifies faint particles (i.e., background noise) as invadopodia, the sensitivity of the invadopodia detection can be decreased by lowering the values for the estimates of minimum and maximum number of invadopodia.

[7] If the "Invadopodia tracker" fails to track invadopodia due to a narrow range between the estimates of minimum and maximum number of invadopodia, then either the value for estimate of minimum number of invadopodia should be decreased or the value for estimate of maximum number of invadopodia should be increased, or both.

[8] The last three parameters in the "Invadopodia tracker" plugin are automatically adjusted according to user-selected values for maximum invadopodium displacement and the estimates of minimum and maximum number of invadopodia. In the case where plugin is not tracking invadopodia properly after adjusting the parameters described in Notes 6 and 7 the last three parameters may be changed to help converge on a solution.

[9] After running the "Invadopodia tracker" plugin, tracked invadopodia are shown in red circles as overlays. In the case a user wants to run the plugin again with different parameters, these overlays can be removed by selecting "Image - > Overlay - > Remove Overlay" command in ImageJ.

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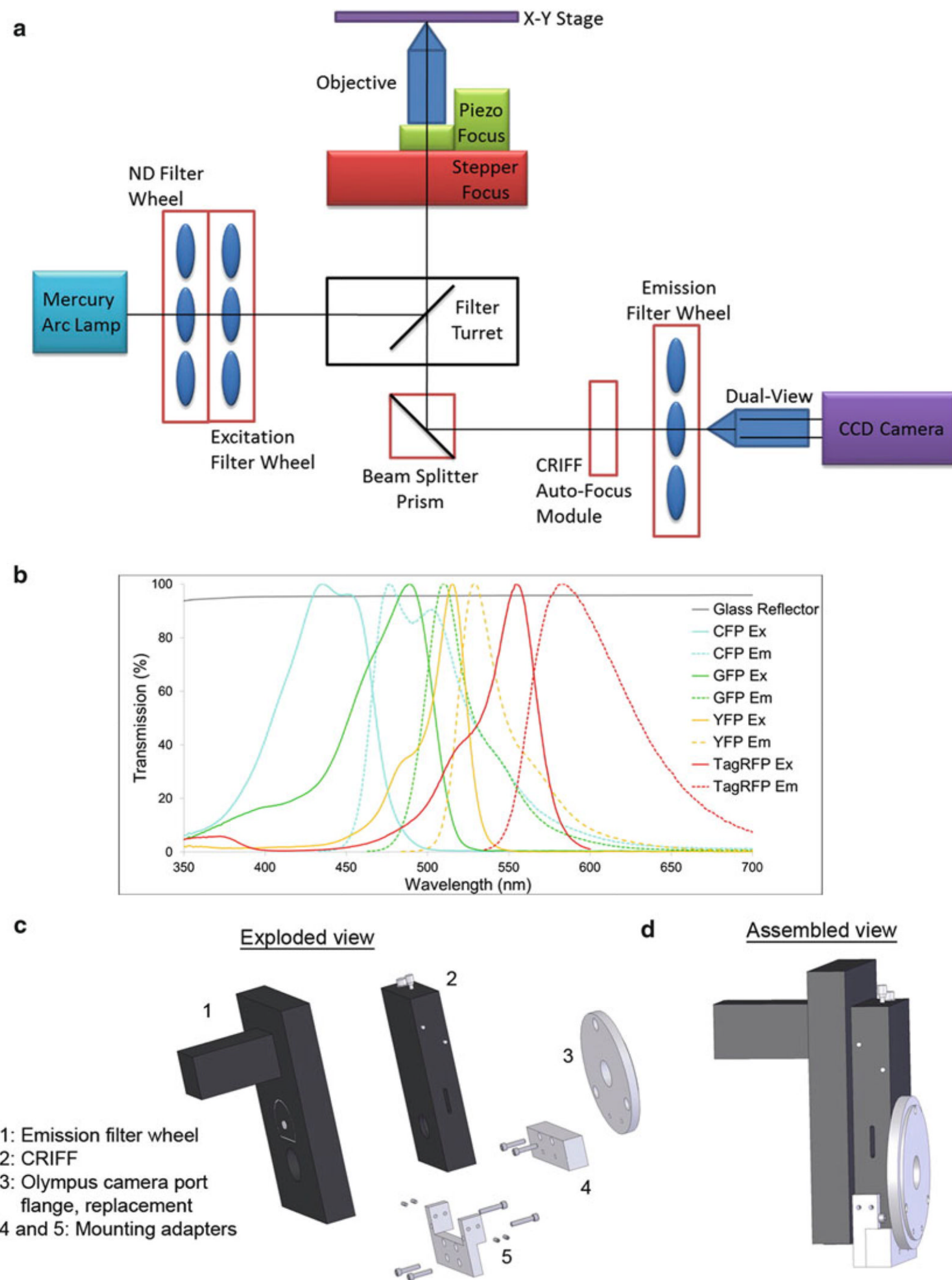

Fig. 1.

Design of custom-built high-resolution wide-field fluorescence microscope. (**a**) Optical layout showing different parts of the microscope. For clarity, the environmental heat chamber, which surrounds the microscope, is not shown. (**b**) Transmission properties of the glass reflector (6 % reflection/94 % transmission) and excitation and emission spectra of commonly used fluorophores in live cell imaging. (**c**) Exploded view of CRIFF, emission filter wheel, flange and the mounting adapters. (**d**) Assembled view of the microscope parts shown in (**c**)

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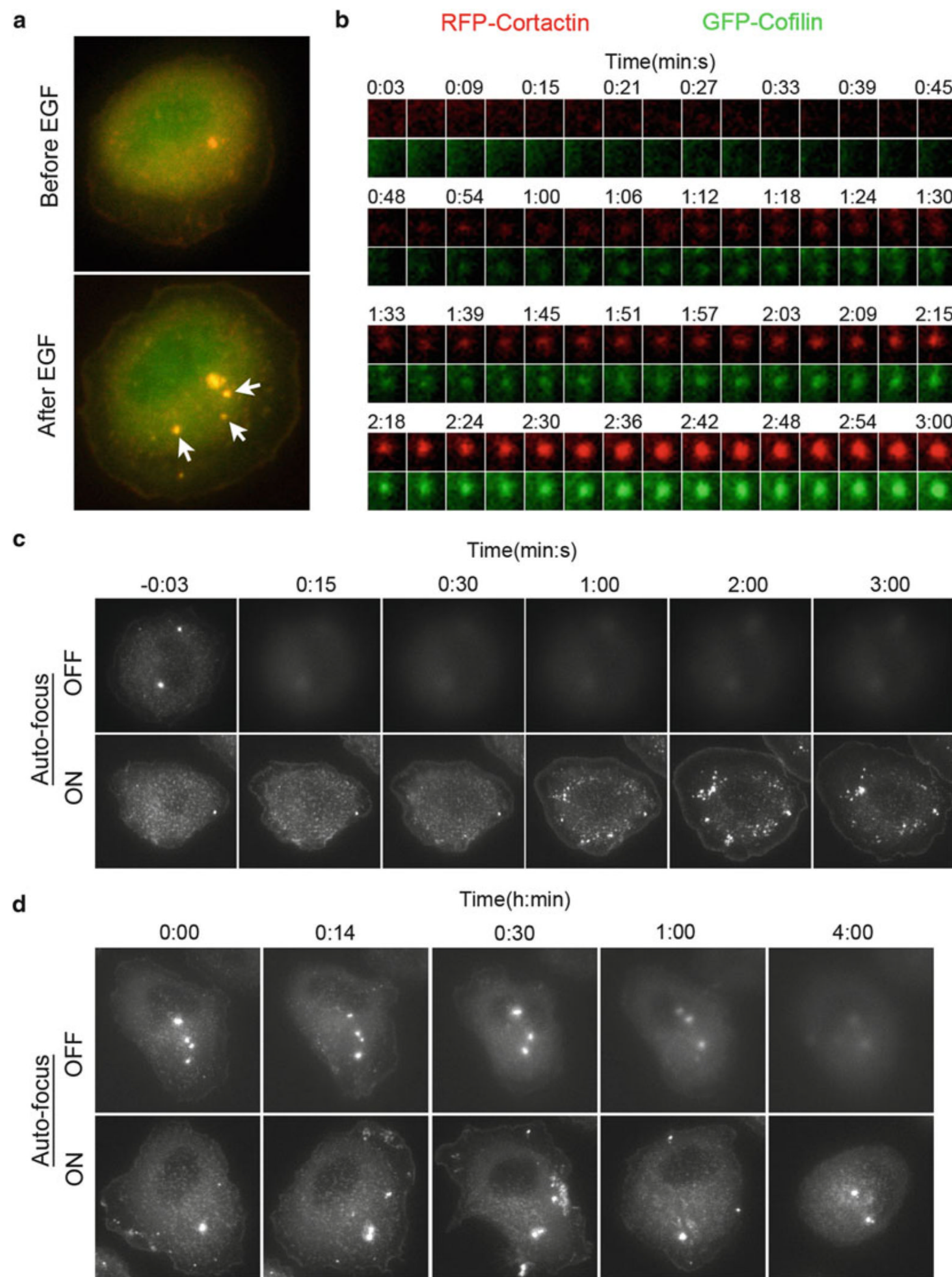

Fig. 2.

Live-cell image acquisition. (**a**) TagRFP-Cortactin and GFP-Cofilin transfected MTLn3 cells were stimulated with EGF and imaged every 3 s for up to 5 min. Images show an MTLn3 cell before and after EGF stimulation. *White arrows* indicate newly forming invadopodium precursors after EGF stimulation. (**b**) Time-lapse sequence of a single invadopodium precursor in GFP and RFP channels is depicted as montage. Time is in min:s. (**c**) Demonstration of the advantage of using autofocus (with CRIFF unit) in maintaining focus during EGF stimulation experiment. Cells expressing TagRFP-Cortactin were imaged with (*lower panel*) or without (*upper panel*) autofocus every 3 s for up to 5 min. Only

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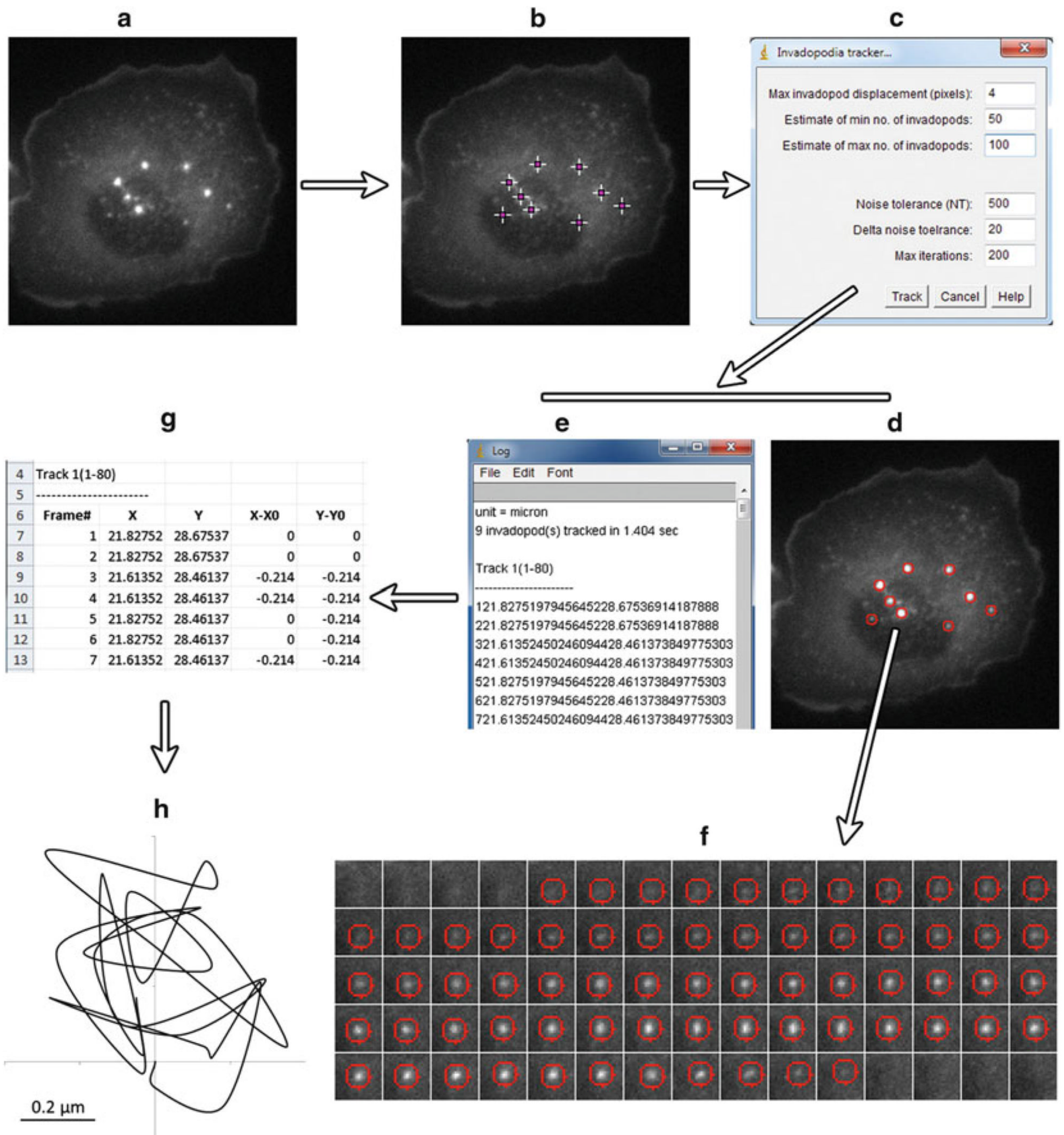
selected time points are shown. Time is in min:s and time 0:00 corresponds to EGF addition. (**d**) Demonstration of the advantage of using autofocus (with CRIFF unit) in maintaining focus during steady state long time-lapse imaging. Cells expressing TagRFP-Cortactin were imaged with (*lower panel*) or without (*upper panel*) autofocus every 2 min for up to 4 h. Only selected time points are shown. Time is in h:min. (**a–d**) In all experiments, an environmental heat chamber enclosing the microscope was preheated to 37 °C for > 3 h, before the start of imaging



4	Track 1(1-80)				
5	----------------------				
6	Frame#	X	Y	X-X0	Y-Y0
7	1	21.82752	28.67537	0	0
8	2	21.82752	28.67537	0	0
9	3	21.61352	28.46137	-0.214	-0.214
10	4	21.61352	28.46137	-0.214	-0.214
11	5	21.82752	28.46137	0	-0.214
12	6	21.82752	28.46137	0	-0.214
13	7	21.61352	28.46137	-0.214	-0.214

Fig. 3.
Flow diagram of invadopodia tracking with the “Invadopodia tracker” plugin in ImageJ. Step-by-step analysis procedure is described in Subheading 3.6.

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Table 1

List of excitation, emission, ND, Turret, and Dual-View filters

Location	Use	Filter	Vendor
Filter Turret Pos. A	Dichroic	FF01-444/520/590	Semrock
	Emission	FF01-465/537/623	Semrock
Filter Turret Pos. B	Dichroic	FF493/574-Di01	Semrock
	Emission	FF01-512/630-25	Semrock
Filter Turret Pos. C	Dichroic	FF660-Di01	Semrock
	Emission	FF01-685/40-25	Semrock
Filter Turret Pos. D	6 % Reflector	6/94bs	Chroma
Excitation Wheel Pos. 1	CFP Excitation	FF01-434/17-25	Semrock
Excitation Wheel Pos. 2	GFP Excitation	FF01-472/30-25	Semrock
Excitation Wheel Pos. 3	YFP Excitation	FF01-500/24-25	Semrock
Excitation Wheel Pos. 4	RFP Excitation	FF01-550/32-25	Semrock
Excitation Wheel Pos. 5	RFP Excitation	FF01-575/25-25	Semrock
Excitation Wheel Pos. 6	Cy5 Excitation	FF01-617/73-25	Semrock
Emission Wheel Pos. 1	Dual View Emission	Open	-
Emission Wheel Pos. 2	CFP Emission	FF01-475/50-25	Semrock
Emission Wheel Pos. 3	GFP Emission	FF01-514/30-25	Semrock
Emission Wheel Pos. 4	YFP Emission	FF01-519-LP	Semrock
Emission Wheel Pos. 5	RFP Emission	FF01-607/36-25	Semrock
Emission Wheel Pos. 6	Cy5 Emission	FF01-685/40-25	Semrock
CFP-YFP DualView Cube	Dichroic	t505lpxr	Chroma
	Short Pass	FF01-475/50-25	Semrock
	Long Pass	FF01-500LP-25	Semrock
GFP-RFP DualView Cube	Dichroic	t540lpxr	Chroma
	Short Pass	FF01-514/30-25	Semrock
	Long Pass	BLP01-532R-25	Semrock
ND Filter Wheel	0.1, 0.2, 0.3, 0.5, 0.6, 1.0, 2.0	nd filter set	Chroma

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Table 2

Microscope imaging modes

Use #	Turret position	Dual-view	Usage
1	A, B, C, D	OFF	Trans-illumination
2	A	OFF	CFP, Cerulean
3	A	OFF	YFP, Venus
4	A	OFF	RFP, mCherry, TRITC, Alexa 555
5	A	OFF	CFP-YFP FRET, Cerulean-Venus FRET
6	B	OFF	GFP, FITC, Alexa 488
7	B	OFF	RFP, mCherry, TRITC, Alexa 555
8	B	OFF	GFP-RFP FRET
9	C	OFF	Cy5, Alexa 647
10	D	OFF	CFP, Cerulean
11	D	OFF	YFP, Venus
12	D	OFF	CFP-YFP FRET, Cerulean-Venus FRET
13	D	OFF	GFP, FITC, Alexa 488
14	D	OFF	RFP, mCherry, TRITC, Alexa 555
15	D	OFF	GFP-RFP FRET
16	D	OFF	Cy5, Alexa 647
17	D	CFP-YFP DualView cube	CFP (Left half) + YFP-FRET (right half)
18	D	CFP-YFP DualView cube	Blank (Left half) + YFP (right half)
19	D	CFP-YFP DualView cube	Blank (Left half) + RFP (right half)
20	D	CFP-YFP DualView cube	Blank (Left half) + Cy5 (right half)
21	D	GFP-RFP DualView cube	GFP (Left half) + RFP-FRET (right half)
22	D	GFP-RFP DualView cube	Blank (Left half) + RFP (right half)
23	D	GFP-RFP DualView cube	Blank (Left half) + Cy5 (right half)