A polarized Ca\(^{2+}\), diacylglycerol and STIM1 signalling system regulates directed cell migration

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Ca\(^{2+}\) signals control cell migration by regulating forward movement and cell adhesion. However, it is not well understood how Ca\(^{2+}\)-regulatory proteins and second messengers are spatially organized in migrating cells. Here we show that receptor tyrosine kinase and phospholipase C signalling are restricted to the front of migrating endothelial leader cells, triggering local Ca\(^{2+}\) pulses, local depletion of Ca\(^{2+}\) in the endoplasmic reticulum and local activation of STIM1, supporting pulsatile front retraction and adhesion. At the same time, the mediator of store-operated Ca\(^{2+}\) influx, STIM1, is transported by microtubule plus ends to the front. Furthermore, higher Ca\(^{2+}\) pump rates in the front relative to the back of the plasma membrane enable effective local Ca\(^{2+}\) signalling by locally decreasing basal Ca\(^{2+}\). Finally, polarized phospholipase C signalling generates a diacylglycerol gradient towards the front that promotes persistent forward migration. Thus, cells employ an integrated Ca\(^{2+}\) control system with polarized Ca\(^{2+}\) signalling proteins and second messengers to synergistically promote directed cell migration.

Migration is a fundamental property of many metazoan cells that enables organisms to develop, repair tissues and defend against pathogens. Cells can move in a directed fashion in response to soluble chemicals or ligands (chemotaxis), mechanical cues (mechanotaxis), and substrate-bound chemo-attractants (haptotaxis)\(^1,2\). Directed migration is often studied in single cells but is also critical for groups of cells that migrate collectively towards an open space or chemoattractant\(^1\). Leader cells at the front of the group respond to environmental stimuli similarly to migrating single cells, whereas follower cells located behind the leader cells migrate on the basis of cues from their neighbouring cells\(^1\).

To move forward and turn, cells require spatial and temporal coordination of force-generating components such as actin and myosin\(^5-7\), as well as regulatory proteins such as Rac, RhoA and Cdc42 (refs 8,9). Nevertheless, how these molecular processes are coordinated for successful cell migration is still incompletely understood.

Ca\(^{2+}\) signals are one such coordinator of cell migration\(^10,11\), partly through local Ca\(^{2+}\) pulses near the leading edge that activate myosin light chain kinase (MLCK) and modulate nascent focal adhesions\(^6,12,13\). Nevertheless, it remains unclear why Ca\(^{2+}\) levels are often lower in the front than in the back of migrating cells\(^11,14,15\), whether receptor tyrosine kinase (RTK), phospholipase C (PLC) or stromal interaction molecule 1 (STIM1) signalling is polarized, whether the co-generated second messenger diacylglycerol (DAG) regulates cell migration in parallel and whether Ca\(^{2+}\) signalling differs between leader cells and follower cells during collective sheet migration.

Many receptor stimuli induce PLC to generate inositol-1,4,5-trisphosphate (Ins\(P_3\)), which activates Ins\(P_3\) receptor in the endoplasmic reticulum (ER), and locally or globally release Ca\(^{2+}\) stored in the ER. Ca\(^{2+}\) signals are terminated by removal of released Ca\(^{2+}\) through plasma membrane (PM) Ca\(^{2+}\) ATPase (PMCA) to the outside, and through ER Ca\(^{2+}\) ATPase (SERCA) back into the ER (ref. 16). PLC also produces the lipid second messenger DAG, which often acts synergistically with Ca\(^{2+}\) in activating cellular processes\(^17,18\). In addition, STIM1 proteins sense low luminal ER Ca\(^{2+}\) and signal across the ER membrane to activate PM Ca\(^{2+}\) influx channels at junctions where the ER contacts the PM.

Here we use live-cell imaging of migrating sheets of endothelial cells to determine if and how this Ca\(^{2+}\) signalling system is spatially organized during migration. We identified gradients in cytosolic and ER Ca\(^{2+}\) levels as well as polarized distributions of growth factor receptor signalling, Ca\(^{2+}\) pulses, DAG, Ca\(^{2+}\) pumps and STIM1, together generating an integrated Ca\(^{2+}\) control system that is uniquely suited to regulate directionality, speed and turning of endothelial leader cells as they move into open space.

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Received 12 July 2013; accepted 10 December 2013; published online 26 January 2014; DOI: 10.1038/ncb2906
RESULTS
Receptor tyrosine kinase signalling is polarized in migrating leader cells

We investigated the collective migration of human umbilical vein endothelial cells (HUVECs) plated as confluent monolayers. Growth factors promote the migration of HUVECs into a band of open space that can be generated by removing cells using a scratch tool\(^1\). In the presence of uniform basic fibroblast growth factor (bFGF), phosphotyrosine signals were higher in the front than in the back of leader cells. In contrast, cells in serum-free medium (SFM) or cells stimulated with bFGF, but inhibited by the pan-tyrosine kinase inhibitor ponatinib\(^2\), lost this phosphotyrosine gradient (Fig. 1a,b and Supplementary Fig. 1a), implying that RTK signalling is polarized. The phosphotyrosine gradient was restricted to leader cells, as it was not observed in follower cells inside the monolayer (Fig. 1b).

We next tested whether PLC, a downstream target of RTK signalling, was also activated in a polarized fashion, using an improved DAG sensor to monitor whether PLC-generated DAG was polarized in migrating cells (protein kinase C, PKC-\(\gamma\)-C1AC1A; Methods). A PM marker was used as a reference (Supplementary Fig. 1b,d). Strikingly, the ratio of membrane-localized DAG sensor over the PM marker was higher in the front versus the back of the cell, implying that a gradient in PLC activation is converted into a gradient in DAG signalling (Fig. 1d,f and Supplementary Fig. 1d). As has been shown in other cells, we used an Akt–PH translocation sensor for phosphatidylinositol 3,4,5-triphosphate (PtdIns(3,4,5)P\(_3\)) to confirm that this co-produced lipid second messenger is polarized\(^3\) (Fig. 1c,e and Supplementary Fig. 1d).

PLC activation also generates InsP\(_3\), which releases Ca\(^{2+}\) from the ER. Indeed, use of a PM-targeted Ca\(^{2+}\) indicator, GCaMP6s–CAAX (ref. 22), revealed a higher frequency of local Ca\(^{2+}\) release pulses in the front than in the back of cells (Fig. 1g and Supplementary Video 1; Methods). Furthermore, these local Ca\(^{2+}\) pulses in the front were dependent on the presence of either bFGF or serum, and were suppressed by ponatinib (Fig. 1h and Supplementary Fig. 1e–h). Together, these data support the interpretation that cyclic local Ca\(^{2+}\) pulses in the front of the cell as well as a gradient of DAG are induced by local RTK-mediated PLC activation (Fig. 1g). These signals and gradients were exclusively observed in leader and not follower cells. Nevertheless, despite the polarized Ca\(^{2+}\) pulses in the front, the average cytosolic Ca\(^{2+}\) levels distributed in an inverted manner, with markedly lower levels in the front than in the back of the cell (Fig. 1i,j), similar to observations in other cell types\(^1,14,15\).

Identification of Ca\(^{2+}\) signalling proteins that regulate collective cell migration

We next conducted short interfering RNA (siRNA) knockdown experiments targeting Ca\(^{2+}\) regulators and used automated microscopy to compare changes in cytosolic Ca\(^{2+}\) level and cell migration speed of leader cells\(^12,19\) (Fig. 2a; Methods). Knocking down the components of the store-operated Ca\(^{2+}\) (SOC) influx pathway, STIM1 and Orai, decreased cytosolic Ca\(^{2+}\) levels, whereas knocking down the Ca\(^{2+}\) pumps PMCA (which pumps Ca\(^{2+}\) out of cells through the PM) and SERCA (which pumps Ca\(^{2+}\) from cytosol into ER) or calmodulin (which regulates PMCA and other Ca\(^{2+}\) regulatory proteins) increased intracellular Ca\(^{2+}\) levels (Fig. 2a,b and Supplementary Fig. 2b). Unexpectedly, these changes in basal Ca\(^{2+}\) were paralleled by opposing changes in migration speed (Fig. 2b). This inverse correlation was confirmed by the overexpression of yellow fluorescent protein (YFP)-conjugated STIM1, which increased basal Ca\(^{2+}\) and at the same time reduced speed (Fig. 2c and Supplementary Fig. 2c). The same opposing effect on migration speed was observed when we increased or decreased basal Ca\(^{2+}\) levels using the SERCA Ca\(^{2+}\) pump inhibitor thapsigargin and the SOC influx inhibitor BTP2 (ref. 23 and Fig. 2d,e). An inhibitory role of local Ca\(^{2+}\) signal pulses\(^2\) was confirmed by observing that loading migrating HUVECs with increasing amounts of the Ca\(^{2+}\)-sensitive fluorescent dye Fura-2 dampened local Ca\(^{2+}\) pulses and increased the speed of migration (Supplementary Fig. 2c; see Methods and Supplementary Fig. 1g and Table 1 for estimation of basal free cytosolic Ca\(^{2+}\) levels).

In order for pulses of Ca\(^{2+}\) to have functional consequences, they must be of sufficiently high amplitude to activate targets, that is by Ca\(^{2+}\) forming a complex with calmodulin and MLCK. We generated small decreases or increases in the cytosolic Ca\(^{2+}\) levels by titrating BTP2 or thapsigargin. HUVECs were then fixed and stained with anti-phospho-myosin light chain (pMLC) antibody to monitor the activity of MLCK. Indeed, we noticed an increase of pMLC staining on small increases in cytosolic [Ca\(^{2+}\)] (Fig. 2f,g). This argues that changes in Ca\(^{2+}\) in the front of less than twofold can activate MLCK and myosin 2. The tight binding of Ca\(^{2+}\)/calmodulin to MLCK (K\(_d\) of 1.1 nM) lowers the required level of Ca\(^{2+}\) needed to activate MLCK (ref. 24), providing a plausible explanation of why small Ca\(^{2+}\) signals are sufficient for activation. In support of this interpretation, treatment with the SOC inhibitor BTP2 decreased local Ca\(^{2+}\) pulses (Supplementary Fig. 2f,g) and nascent focal adhesions (Fig. 2h,i and Supplementary Fig. 2h,i) with a rate similar to that caused by the myosin 2 inhibitor blebbistatin. Thus, small Ca\(^{2+}\) pulses have a critical physiological role in the cyclic regulation of local MLCK activity in the front of migrating HUVECs.

The relative strength of cell–matrix adhesion determines whether STIM1 enhances or suppresses the speed of migration

Our result for the effect of STIM1 knockdown on HUVEC migration speed differed from previous studies carried out in cancer cells\(^27,28\) and smooth muscle cells\(^27,28\), which showed reduced migration following STIM1 knockdown. We verified STIM1 knockdown by western blotting (Supplementary Figs 2a and 3g) and further validated our findings by carrying out rescue experiments, where the effect of STIM1 depletion was reversed by overexpression of exogenous STIM1, and where BTP2 treatment abrogates rescue by exogenous STIM1 (Supplementary Fig. 2c,d). STIM1 knockdown using the same siRNAs also had a small but opposing effect on migration of H1299 metastatic lung cancer cells when compared with HUVECs (Fig. 3a).

A possible reconciliation between the opposite results in the two cell types is the role of SOC and local Ca\(^{2+}\) pulses in enhancing the assembly rate of focal adhesion complexes, as shown here (Fig. 2h,i) and in previous studies\(^12,29\). Although cell–matrix adhesion is critical for cell migration, strong cell–matrix adhesion can impede cell motility\(^30\). Therefore, increased cell–matrix adhesion might have contrasting effects on cell migration in weakly (metastatic cancer cells) versus strongly (HUVECs) adherent cell types. Consistent with this hypothesis, cell migration speed for H1299 cells was higher
Figure 1 RTK signalling is restricted to the front of migrating leader cells. 
(a,b) bFGF-induced tyrosine phosphorylation was higher in the front of migrating cells (white arrows). Addition of the pan-RTK inhibitor ponatinib blocked tyrosine kinase signalling in the front, but not in the back, of leader cells. Follower cells did not respond to bFGF. HUVECs were fixed and stained with PY20 anti-phosphotyrosine antibody (n = 107, 105, 115, 110 and 107 cells for SFM, follower cells and ponatinib 0 nM, 25 nM and 100 nM, respectively). A.U. (0-65535) is derived from the original signal values of the 16-bit images. 
(c,d) Migration ratio images of leader cells co-expressing YFP–Akt–PH (PtdIns(3,4,5)P3) sensor and YFP–C1AC1A (DAG sensor) and a PM marker (CFP–mCD4). PtdIns(3,4,5)P3 (c) and DAG (e) were enriched in the front of migrating cells. Front-to-back gradients of PtdIns(3,4,5)P3 and DAG were present in leader, but not follower cells (24 leader and 42 follower cells in e, 28 and 62 cells in f). 
(g) 

HUVECs were measured as relative increases in local PM-targeted GCaMP6s fluorescence intensity. Higher activities were observed in the front (1) when compared with the middle (2) or back (3) of migrating cells. (h) Relative mean amplitudes of local Ca2+ fluctuations measured over 3 min in the front of migrating cells in response to serum or serum plus ponatinib (see also Supplementary Fig. 1e,f). Amplitudes of Ca2+ fluctuations were normalized to basal cytosolic levels (0.3 relative units (R.U.) means the fluctuation is 30% of the average cytosolic [Ca2+] level; n = 24 cells). (i,j) Migrating HUVECs expressing GCaMP6s–CAAX and the reference membrane marker mCD4 were used to measure Ca2+ gradients in leader and follower cells (n = 83 leader and n = 86 follower cells). Bars denote mean ± s.e.m. in b,h. A Student t-test was used for b,e,f,h,j. In e,f,j, P-values were calculated by comparing the ratio of the sensor/PM intensity ratios in the front and back (both regions were 10% of cell length).
Figure 2 SOC influx controls cell migration by regulating cell–matrix adhesion in the front of migrating cells. (a) HUVEC migration into open space was monitored by staining cells with CellMask (Methods). Accelerated sheet migration was observed in STIM1-depleted when compared with control cells. (b) Comparing changes in the rate of sheet migration and cytosolic Ca^{2+} levels in HUVECs treated with siRNAs targeting different Ca^{2+} signalling regulators. Average cytosolic [Ca^{2+}] was normalized. 1 R.U. equals the level of cells treated with control siRNA (n = 4 experiments for each siRNA). (c) Reduced single cell migration speed in cells overexpressing YFP–STIM1. Cells expressing YFP–ER were used as control (n ~ 10,000 cells per condition). (d,e) Effects of the ER Ca^{2+} pump blocker thapsigargin (d) and the SOC inhibitor BTP2 (e) on cytosolic Ca^{2+} levels and on sheet migration speed. Notice that increasing cytosolic Ca^{2+} levels with thapsigargin decreased migration speed, and lowering Ca^{2+} levels with BTP2 increased migration speed (n = 4 experiments per condition). (f,g) Migrating HUVECs were treated with different concentrations of BTP2 or thapsigargin to reduce or elevate cytosolic Ca^{2+} levels. Cells were then fixed and stained with anti-pMLC antibody. (f) pMLC signals were lower when SOC was blocked by BTP2 but higher when ER Ca^{2+} pumps were blocked by thapsigargin. CAAX, PM marker. (g) pMLC levels increased with increasing cytosolic [Ca^{2+}] (n = 123, 134, 127, 126, 123, 117 and 142 cells per condition from left to right). (h,i) Effect of BTP2 treatment on cell–matrix adhesion. Focal adhesion formation was monitored by expressing GFP–paxillin. BTP2 treatment rapidly decreased the intensities of GFP–paxillin puncta in the front of migrating cells, consistent with SOC influx promoting cell–matrix adhesion. Bars are mean ± s.e.m. in b,d,e,g.
Figure 3 SOC increases migration speed when cell–matrix adhesion is weak, but slows down migration when adhesion is strong. (a) Knocking down STIM isoforms increased sheet migration speed in HUVEC (left) but caused a small and significant reduction in speed in H1299 cells (right; \( n = 4 \) experiments per condition). (b) H1299 cells migrated faster on high fibronectin. BTP2 treatment decreased migration speed of H1299 cells on low fibronectin or when treated with BTP2. (c) Overexpression of YFP–STIM1 in H1299 cells (1, 2) on low fibronectin increased cell substrate adhesion (paxillin staining) when compared with control cells (3). (f) Average paxillin puncta intensity for control cells and for cells expressing high levels of YFP–STIM1 (\( n = 42 \) and 23 cells from left to right per condition). (g) Single cell speed in sheet migration as a function of YFP–STIM1, mCitrine–paxillin or YFP–ER overexpression in H1299 cells. Overexpression of YFP–STIM1 and mCitrine–paxillin accelerated sheet migration on low fibronectin but decreased migration speed on high fibronectin. Solid lines and dashed lines are mean \( \pm \) s.e.m.; \( n = 4,714 \) (red) and 4,538 (blue) cells with STIM1, 3,873 (red) and 4,327 (blue) cells with paxillin, and 5,698 (red) and 5,368 (blue) with ER marker. The \( P \)-value compares the blue and red groups for signals for different treatments in c (\( n = 115, 121 \) and 104 cells from left to right per condition). (e) Overexpression of YFP–STIM1 in H1299 cells (1, 2) on low fibronectin increased cell substrate adhesion (paxillin staining) when compared with control cells (3).

High fibronectin than on low fibronectin, whereas BTP2 decreased focal adhesions in both conditions (Fig. 3c,d and Supplementary Fig. 3a,b). Cells overexpressing STIM1 protein also formed more focal adhesions (Fig. 3e,f and Supplementary Fig. 3c–f), indicating that SOC is sufficient to enhance focal adhesion. Finally, both STIM1 and paxillin overexpression enhanced migration speed for H1299 cells on low fibronectin.
fibronectin but decreased speed for cells on high fibronectin (Fig. 3g). Thus, STIM1 and SOC-mediated Ca\(^{2+}\) signals enhance matrix adhesion, which in turn either accelerates or slows cell migration depending on whether adhesion is weak or strong, respectively.

**STIM1 is polarized to the front of migrating cells by microtubule plus-end-based transport**

We discovered that expressed YFP–STIM1 was significantly enriched in the front of migrating leader cells (HUVECs) when compared with a cyan fluorescent protein (CFP)-conjugated ER marker protein (Fig. 4a–c and Supplementary Fig. 4a). STIM1 can be transported within the ER membrane by binding to the microtubule plus-end protein EB1 (ref. 31). To test whether microtubule-mediated transport was responsible for the polarization of STIM1, we expressed a YFP-conjugated EB1-binding deficient mutant STIM1 (Fig. 4d). YFP–S1NN increased overall SOC influx to a similar degree as wild-type YFP–STIM1 (YFP–S1wt), indicating that the mutant maintained its full ability to control Ca\(^{2+}\) influx (Supplementary Fig. 4b,c). However, YFP–S1NN failed to polarize in migrating cells (Fig. 4e), implying that wild-type STIM1 is actively transported by microtubule plus ends to the front and that microtubule-based targeting is important for STIM1 localization but not its activity. Nonetheless, HUVECs expressing high levels of wild-type STIM1 markedly decreased their motility, whereas cells expressing similar levels of YFP–STIM1–S1NN showed a smaller reduction in speed (Fig. 4f). This suggests that the localization of STIM1 towards the front is important for its role in cell migration.

Because STIM1 only regulates SOC after its localization to ER–PM junctions, localization of STIM1 at ER–PM junctions can be used as an indicator for SOC influx. We expressed YFP-tagged STIM1 together with a CFP-tagged marker for ER–PM junctions in migrating HUVECs. The ER–PM marker includes a single ER membrane-spanning region, a cytoplasmic linker that is several nanometres long, and a polybasic PM interaction domain (Methods). Confocal microscopy images focused at the bottom membrane of the cells showed enhanced accumulation of YFP–STIM1 at ER–PM junction sites in the front compared with the back, implying that not only is STIM1 polarized towards the front but, in addition, there is enhanced STIM1 activity to activate SOC in the front (Fig. 5a–d and Supplementary Fig. 4d). In support of this interpretation, the S1NN STIM1 mutant, even though it was evenly distributed within the ER (Fig. 4e), was also enriched at ER–PM junctions in the front (Fig. 5c).

**A gradient in the level of Ca\(^{2+}\) in the lumen of the ER of polarized HUVECs**

Because STIM1 localization to ER–PM junctions is directly regulated by ER luminal Ca\(^{2+}\) levels, we tested if the ER luminal Ca\(^{2+}\) level is lower in the front. An ER luminal Ca\(^{2+}\) indicator for SOC influx. We expressed YFP-tagged STIM1 together with a CFP-tagged marker for ER–PM junctions in migrating HUVECs. The ER–PM marker includes a single ER membrane-spanning region, a cytoplasmic linker that is several nanometres long, and a polybasic PM interaction domain (Methods). Confocal microscopy images focused at the bottom membrane of the cells showed enhanced accumulation of YFP–STIM1 at ER–PM junction sites in the front compared with the back, implying that not only is STIM1 polarized towards the front but, in addition, there is enhanced STIM1 activity to activate SOC in the front (Fig. 5a–d and Supplementary Fig. 4d). In support of this interpretation, the S1NN STIM1 mutant, even though it was evenly distributed within the ER (Fig. 4e), was also enriched at ER–PM junctions in the front (Fig. 5c).
cells when compared with the back (Fig. 5e,f). Because local Ca\(^{2+}\) pulses are more frequent in the front than the back of migrating cells (Fig. 1g and Supplementary Video 1), the lower level of luminal ER Ca\(^{2+}\) in the front was probably a result of local InsP\(_3\)-mediated Ca\(^{2+}\) release\(^{16}\). In support of this hypothesis, the pan-RTK inhibitor ponatinib reduced the translocation of STIM1 to ER–PM junctions in the front of migrating cells (Supplementary Fig. 4g–i).

**Low basal Ca\(^{2+}\) in the front of migrating cells is maintained by enhanced pump activity of the PMCA**

Our previous\(^{12}\) and present studies have shown that MLCK is dynamically regulated by local Ca\(^{2+}\) pulses near the front of migrating cells. Therefore, cells have to maintain a basal Ca\(^{2+}\) level in the front below a critical threshold to prevent persistent MLCK activation (Fig. 2fg and refs 12,24). Experiments with the Ca\(^{2+}\) indicator Fura-2 (Fig. 6a) confirmed (Fig. 1i) that HUVECs exhibit a Ca\(^{2+}\) gradient with lower basal Ca\(^{2+}\) levels in the front. This gradient is reduced by increased buffering of Ca\(^{2+}\) when we increase Fura-2 in the cytosol (Fig. 6b). When we elevated cytosolic Ca\(^{2+}\) levels using the SERCA inhibitor thapsigargin\(^{35}\), the previously reported correlation between local Ca\(^{2+}\) pulses and lamellipodium retraction\(^{12}\) was lost (Supplementary Fig. 5a,b). This suggests that low basal Ca\(^{2+}\) levels in the front are critical for cyclic regulation of lamellipodium retraction and adhesion by Ca\(^{2+}\) pulses.

To attain low cytosolic Ca\(^{2+}\) in the front, cells may pump Ca\(^{2+}\) either more strongly into the ER through SERCA pumps, or, alternatively, out of the cell through PM-localized Ca\(^{2+}\) pumps (PMCA). Polarized SERCA pump activity was probably not involved in setting up the gradient because inhibition of SERCA using thapsigargin increased rather than decreased the Ca\(^{2+}\) gradient (Supplementary Fig. 5c,d). However, inhibition of PMCA in migrating cells using either the inhibitor caloxin 2A1 or La\(^{3+}\) reduced the Ca\(^{2+}\) gradient (Fig. 6c) whereas overall cytosolic Ca\(^{2+}\) levels were elevated (Supplementary Fig. 5e) and sheet migration was decreased (Supplementary Fig. 6c).
In a second protocol, we simultaneously treated migrating cells with the SERCA inhibitor thapsigargin and the SOC inhibitor BTP2 and also chelated external Ca\(^{2+}\) using EGTA. This protocol immediately released Ca\(^{2+}\) from the ER into the cytosol, followed by a slower removal of Ca\(^{2+}\) from the cytosol to the extracellular space. The time course of the change in local Ca\(^{2+}\) level (the slope in Fig. 6d) during the removal phase can be used to derive the local Ca\(^{2+}\) pump rate of PMCA (pump rate over local cell volume). A comparison of the local Ca\(^{2+}\) level (x axis) and the local Ca\(^{2+}\) pump rate (y axis) for each time showed that the pump activity (slope in Fig. 6e) in the front of migrating cells is significantly higher than in the back (Fig. 6e,f). The same differential pump activity was confirmed by uniformly releasing Ca\(^{2+}\) using a ultraviolet pulse from an intracellular nitrophenyl EGTA (NP-EGTA) Ca\(^{2+}\) buffer, which induced a cell-wide transient Ca\(^{2+}\) spike (Fig. 7a–d). Furthermore, the differential pump activity could be eliminated by inhibitors of PMCA (Fig. 6g and Supplementary Fig. 5f), but not by inhibitors of Na\(^{+}\)–Ca\(^{2+}\) exchangers, known to contribute to basal Ca\(^{2+}\) homeostasis (Fig. 7c–d and Supplementary Fig. 6a,b). This confirmed a specific role of PMCA in generating Ca\(^{2+}\) gradients in HUVECs.

A gradient in PMCA pump rate could be mediated either by differential regulation of local PMCA activity, by differences in the surface-to-volume ratio between the front and the back or by there being more relative PMCA in the front when compared with the back. We observed a polarized distribution of green fluorescent protein (GFP)-tagged PMCA4, an isoform that is prominently expressed in the front, and this higher rate is at least in part the result of a polarized distribution of PMCA. A gradient in DAG is necessary for effective directed migration

When we treated migrating cells with the PLC inhibitor U73122, the DAG gradient observed in Fig. 1 disappeared (Fig. 8a). To clarify if the DAG gradient was the result of a gradient of its precursor PtdIns(4,5)P\(_2\), we monitored the distribution of PtdIns(4,5)P\(_2\) using the PtdIns(4,5)P\(_2\) sensor PH–PLC\(_6\). Ratio imaging of PH–PLC\(_6\) and a PM marker showed no significant difference in PtdIns(4,5)P\(_2\) distribution.
Figure 7 Higher Ca\textsuperscript{2+} pump activity in the front when compared with the back generates a gradient of basal [Ca\textsuperscript{2+}] in migrating cells. (a–d) Ultraviolet flash photolysis experiments confirmed differential Ca\textsuperscript{2+} pump activities in migrating HUVECs as shown in Fig. 6c–e. (a) Migrating HUVECs were pre-loaded with Fluo-3 AM and NP-EGTA. Thapsigargin and EGTA were added 10 min before imaging to block the activity of SERCA and inlux Ca\textsuperscript{2+} channels. An ultraviolet pulse was used to induce a Ca\textsuperscript{2+} spike 1 min after recording began. (b,c) Ca\textsuperscript{2+} pump activities (k) in the front and in the back were calculated on the basis of Fluo-3 measurements following ultraviolet photolysis, similar to Fig. 6d,e. (d) Quantiﬁcation of relative Ca\textsuperscript{2+} pump activities in the front when compared with the back generates a gradient of basal [Ca\textsuperscript{2+}] in migrating cells. (e) Inhibitors of Na\textsuperscript{+}–Ca\textsuperscript{2+} exchangers 2,4-dichlorobenzene (DCB) and 3,4-DCB both caused a small increase in cytosolic Ca\textsuperscript{2+} levels as measured by Fura-2. EGTA, thapsigargin (Th) and BTP2 were used as positive controls.

(Supplementary Fig. 1c,d), further supporting the interpretation from Fig. 1 that migrating leader cells establish a gradient in DAG from front to back by inducing locally higher PLC activity in the front.

However, treatment of HUVECs with the PLC inhibitor U73122 slowed the speed of migration (Fig. 8b and Supplementary Fig. 7a), which was opposite to the expected result because PLC inhibition suppresses Ca\textsuperscript{2+} signalling, which we showed accelerates migration (Fig. 2a–e). This raised the possibility that the co-generated gradient in DAG has an important parallel role in enhancing the speed of cell migration. In support of such a role of DAG, we observed that high expression of the DAG binding translocation sensor YFP–PKC–C1AC1A caused a dose-dependent decrease in the
Figure 8 A PLC-induced gradient of DAG controls cell motility and directionality. (a) Addition of the PLC inhibitor U73122 (1 μM) suppressed the DAG sensor accumulation observed in the front of control cells (n = 64 cells for control; n = 22 cells for U73122). (b) The PLC inhibitor U73122 slowed down the single cell speed of HUVECs as well as sheet migration speed in a dose-dependent manner (Supplementary Fig. 7a). U73122 was added to the cell sheets before time-lapse imaging (n = 2 experiments for each group). (c–e) Overexpression of YFP-C1AC1A reduced the measured migration parameters of directionality, single cell speed and directional persistence. (c) Cell migration traces of 50 randomly chosen cells not expressing C1AC1A (left) and 50 cells overexpressing C1AC1A (right) are shown. The traces were aligned to start at the origin (0 μm, 0 μm) with the direction of the wound at the top. Cells expressing C1AC1A had relatively traces and partially lost their orientation towards the open space. (d) Schematic diagram showing how the migration parameters of speed and directionality shown in e were determined. (e) Statistical analysis of the change in directionality, speed and persistence in response to YFP-C1AC1A overexpression calculated for each migrating cell and correlated with binned levels of YFP-C1AC1A expression. Increasing YFP-C1AC1A expression resulted in decreasing directionality, speed and persistence. Bars are mean ± s.e.m. (n = 1,200 cells). (f) Partial inhibition of PKC by ruboxistaurin decreased the rate of sheet migration. The reduction could be rescued by inhibiting DAG kinase using two types of inhibitor. Bars are mean ± s.e.m. (n = 3 experiments per condition). (g) Schematic representation of the identified gradients in the Ca²⁺ and DAG signalling system. In a, P-values were calculated with a Student t-test on the basis of the ratio of the sensor/PM ratio in the front 10% region to that in the back 10% region of migrating cells. One-way ANOVA was used for b,e,f to determine the significance of differences between multiple groups.
speed and directionality of cell migration (Fig. 8c–e), probably caused by a dominant negative effect whereby the DAG biosensor partially sequesters PM DAG. Furthermore, application of two different DAG kinase inhibitors that increase the intracellular concentration of DAG (ref. 40) both increased the speed and persistence of leader cells in a concentration-dependent manner (Supplementary Fig. 7b).

Because PKC is a main intracellular target of DAG and because PKCs have a role in regulating actin polymerization by phosphorylating actin regulators such as myristoylated alanine-rich protein kinase C substrate (MARCKS), adducin, fascin and ERM proteins, we tested whether DAG enhances directed cell migration through PKC. Indeed, addition of the PKCβ inhibitor ruboxistaurin decreased the sheet migration speed in a concentration-dependent manner (Supplementary Fig. 7c, leftmost bars). Interestingly, at intermediate concentrations of ruboxistaurin, inhibition of DAG kinase restored migration (Fig. 8f and Supplementary Fig. 7c), consistent with the interpretation that DAG signals through PKCβ to promote directed cell migration. Together, this suggests that local RTK signalling in the front causes local PLC activation and DAG production, which in turn selectively activates PKCβ in the front.

**DISCUSSION**

Our study shows that migrating endothelial leader cells establish a gradient in basal free Ca\(^{2+}\) levels with Ca\(^{2+}\) levels being lowest in the front. This is at least in part a result of higher localized PM Ca\(^{2+}\) pump activity in the front (Supplementary Fig. 8e and Fig. 7g,h) and is facilitated by a slow diffusion coefficient of Ca\(^{2+}\) (\(D = 10\ \mu\text{m}^2\ \text{s}^{-1}\), reduced by Ca\(^{2+}\) buffers)\(^4\) and a relatively extended length of HUVECs of \(x_0 \sim 55 \pm 16\ \mu\text{m}\) (Supplementary Fig. 7d). Given these parameters, the diffusion-mediated equilibration time of \(x_0^2/(2D) \sim 150\) s is longer than the time required for Ca\(^{2+}\) pump-mediated Ca\(^{2+}\) extrusion, enabling a Ca\(^{2+}\) gradient to form (Fig. 1i,h and Fig. 6a). We further identified a parallel gradient of Ca\(^{2+}\) levels in the lumen of the ER (Fig. 5e,f) that we could explain by bFGF (refs 4,19,43) signalling being restricted towards the front of migrating leader cells and generating local PLC activation (Fig. 1a–f). We also showed that RTK and PLC signalling as well as Ca\(^{2+}\) gradients are largely absent from follower cells inside the sheet (Fig. 1b). The absence of significant RTK signalling both in the back of leader cells and in the front and back of follower cells suggests that cell–cell contacts may locally suppress receptor signalling.

Localized receptor signalling towards the front has two major consequences; it explains the small localized cyclic InsP\(_3\)–triggered Ca\(^{2+}\) release pulses (Supplementary Fig. 1e,f) and the establishment of a DAG gradient (Fig. 1d). While little was previously known about the existence of gradients in DAG during migration, local DAG signals have been observed in pollen tube germination in plants\(^{44,45}\), after activation of T-cell receptors\(^{46-48}\) during phagocytosis\(^{49-52}\), and in neuronal synapses to regulate secretion of neurotransmitters\(^{53,54}\). Finally, we show that STIM1 is activated locally in the front of migrating cells (Fig. 4) as a result of directed STIM1 transport to the front mediated by microtubule plus-end transport and lower ER Ca\(^{2+}\) levels in the front mediated by local RTK signalling. The resulting polarized SOC signalling provides a key mechanism to maintain the spatial and temporal dynamics of the Ca\(^{2+}\) signalling system.

The functional relevance of our study builds on previous findings on the roles of MLCK in membrane retraction\(^{55}\) and the role of STIM1 (refs 25–29) in regulating cell migration and adhesion. Here we investigated whether Ca\(^{2+}\), DAG and STIM1 act in a polarized fashion and have synergistic roles in regulating directed migration. We show that microtubule plus-end–mediated transport of STIM1 to the front and that local STIM1 activation in the front is critical for its role in regulating migration. Our study argues that STIM1 probably acts indirectly on adhesion by enhancing local Ca\(^{2+}\) influx and by reloading ER Ca\(^{2+}\) stores in the front to permit local Ca\(^{2+}\) pulses to be cyclically triggered and MLCK to be locally activated. It is interesting that our study identified an opposite effect of SOC influx on cell migration when compared with previous studies in cancer cells\(^{25-26}\). We were able to explain this discrepancy by showing that the relative strength of cell–matrix adhesion can decide whether SOC influx and further adhesion decelerates or accelerates migration.

Our study further argues that the polarization of DAG that we discovered selectively activates the actin machinery in the front to enhance forward movement and to promote a more persistent polarized migration state. While several actin regulatory proteins, including classic and new PKC, protein kinase D and Ras guanyl releasing proteins (RasGRPs) (refs 45,55), are activated by DAG, our kinase inhibitor data suggests that the classical PKC pathway, which relies on combined Ca\(^{2+}\) and DAG signals, is significantly involved. Previous studies have shown that PKCs can regulate migration\(^56\), probably in a synergistic fashion, by phosphorylating myosin\(^57\), by regulating the actin cytoskeleton\(^41\) or by the turnover of integrin complexes\(^58\). Together with our finding of DAG polarization, this suggests that selective DAG signalling and PKC activation of actin regulators in the front promotes polarization and persistence of migration.

In summary, our study provides an integrated model of the spatial organization of the PLC-Ca\(^{2+}\)–DAG–STIM1 signalling system. We show that core Ca\(^{2+}\) system components are polarized in migrating endothelial leader cells, including upstream receptor signalling, local Ca\(^{2+}\) pulses, DAG, Ca\(^{2+}\) pumps, basal cytosolic and ER Ca\(^{2+}\) levels as well as STIM1 distribution and STIM1–mediated SOC influx (Fig. 8g and Supplementary Fig. 8). Together with the actin regulators Rac, Cdc42, RhoA and PtdIns(3, 4, 5)P\(_3\), this Ca\(^{2+}\) and DAG regulatory system dynamically controls the polarization and persistence of migration as well as local adhesion and turning.

**METHODS**

Methods and any associated references are available in the online version of the paper.

*Note: Supplementary Information is available in the online version of the paper*

**ACKNOWLEDGEMENTS**

We thank S. R. Collins, M. Galic and R. Wollman for technical support and discussions, S. Bandara for the T1ER construct, N. Borghi for the paxillin construct, X. Ge for the CD4 construct, C. J. Lin for H1299 cells, E. E. Strehler for the PMCA constructs and A. Winans for critical reading of the manuscript. The research was supported by a Stanford Graduate Fellowship (F.-C.T.) and the NIGMS (T.M.)

**AUTHOR CONTRIBUTIONS**

F.-C.T. conceived, designed and carried out experiments, analysed the data and wrote a draft of the manuscript. A.S. developed the DAG sensor and helped with the DAG–related experiments and western blotting, H.W.Y. repeated and validated...
experiments with the lipid sensors, and compared gradients in leader and follower cells. A.H. helped generate the paullin constructs, prepared cells stably expressing reference membrane markers and helped write the manuscript. S.C. developed the ER–PM and ER-membrane markers. S.M. developed the membrane-targeted version of GCaMPs and helped with ruboxuturin experiments. T.M. conceived the project together with F.C.T., and helped interpret the data and write the manuscript.

COMPETING FINANCIAL INTERESTS
The authors declare no competing financial interests.

Published online at www.nature.com/dofinder/10.1038/rcb2906
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METHODS

Cell culture. HUVECs (Lonna, C2519A) were cultured in an EGM2 (Lonna, CC-3162) kit, as described previously14. HUVECs were tested to be free from mycoplasma by H.W.Y. and A.H. H1299 cells (provided by C. P. Chang, Stanford University) were cultured in DMEM supplemented with 10% FBS, 1% penicillin and 1% streptomycin. For migration experiments, glass bottom 96-well plates (Greiner) were coated with fibronectin (Life Technologies) at concentrations ranging from 0.31 μg to 20 μg ml⁻¹ in 1× PBS (GIBCO) for 2 h. H1299 cells were plated at 50,000 cm⁻² (for single cell tracking) or 100,000 cm⁻² (for sheet migration assay) 12 h before experiments.

Reagents and dyes. Fura-2 AM, Fluo-3 AM, thapsigargin, BFGF and CellMask membrane dye were purchased from Invitrogen. BTP2 was purchased from Calbiochem, potentiobin from Selleck, U71322 from Tocris, DAG kinase inhibitor I, DAG kinase inhibitor II, 3,4-DCB and LacI, from Sigma Aldrich, robuxostatin from AG Scientific, caloxin 2A1 from AnaSpec and 2,4-DCB from Santa Cruz. Anti-phosphotyrosine monoclonal antibody was from Santa Cruz (PY20, sc-508) and was used at 1:500 dilution for immunofluorescence. Paxillin:FITC (fluorescein isothiocyanate) antibody was purchased from BD Biosciences (P15324-050/610053, mouse IgG1 clone 349) and used at 1:100 for immunofluorescence. Anti-pMLC2 (Ser 19, no 3671, rabbit) was purchased from Cell Signaling and used at 1:500 for immunofluorescence.

siRNA and DNA constructs. In vitro dicer siRNA pools were generated as described previously2,15. In brief, a ~600 bp (base-pair) DNA fragment selected from the coding region of each gene was amplified by two nested polymerase chain reactions using human complementary DNA as template and primers with T7 promoters. Sequences of primers for nest polymerase chain reaction are available in Supplementary Table 2. Double-stranded RNA was then generated by in vitro transcription using T7 polymerase and treated with Giardia Dicer to produce fragments of 24–27 bp. A synthetic pool of siRNAs targeting STIM1 was purchased from Dharmacon (MU-011785-00-0002). mCD4 (labelled as YFP and mCD4) N1 construct was derived from pECFP N1 (Clontech). Primers for

Ca²⁺ measurements. Fura-2 AM or GCaMP6s was used to measure cytosolic Ca²⁺ in HUVECs. For loading of Fura-2 AM could not be used. TIER (ref. 33,34), an enhanced version of the DIER construct, was used to measure luminal ER Ca²⁺ (Addgene plasmid no 47928). GCaMP6s–CAAX or TIER were transiently transfected into HUVEC 1 day before the experiments. Dye loading was done as follows. Fura-2 AM and Fluo-3 AM were loaded at 0.3–2 μM with 0.1% Pluronic F-127 (Invitrogen) and 1 μM probenecid (Invitrogen) in endothelial SFM (GIBCO) at 37 °C for 30 min. The cytosolic Ca²⁺ was then measured and calibrated as described previously22. In brief, images were taken using ×20 Plan Fluar (ImageXpress microscope) or ×40 Neofluar (Nikon microscope) objectives and epifluorescence illumination, with excitation wavelengths of 340 and 380 nm for Ca²⁺-bound and Ca²⁺-free dyes, respectively, and an emission wavelength of 510 nm. Relative Ca²⁺ levels were determined on the basis of the ratio between the images acquired using 340 and 380 nm excitation. For calibration, a set of solutions with Fura-2 and various concentrations of CaCl₂ (ranging from 0–1,000 nM) was used. Fura-2/CaCl₂ solutions were made by mixing Ca²⁺ EGTA and KEGTA, as previously described22. The calibration curve is shown in Supplementary Fig. 1e. The estimated Kₐ of Fura-2 was 101 nM. Because the Kₐ of Fura-2 is affected by ionic strength, sub-cellular localization, protein binding and temperature23,24, the actual intracellular Kₐ may be higher. Therefore, we included estimates for absolute intracellular [Ca²⁺]ᵢ using also the Kₐ of 145 nM from the manufacturer25, and 224 nM from ref. 66 (Supplementary Table 1). Depending on the Kₐ values, the estimated basal cytosolic Ca²⁺ level falls between 45 and 105 nM in HUVECs, compatible with the measurement from previous reports26,27.

As indicated above, there are challenges for measuring absolute intracellular [Ca²⁺]ᵢ in HUVECs. Therefore, instead of using absolute [Ca²⁺]ᵢ values, we included R.U. by normalizing [Ca²⁺]ᵢ to the measured average basal cytosolic level. 1 R.U. means the Ca²⁺ level is close to the average cytosolic [Ca²⁺]ᵢ, and 2 R.U. means the Ca²⁺ level is approximately twice as high as the basal cytosolic [Ca²⁺]ᵢ. In some cases we used the Fura-2 340/380 ratio where appropriate (Fig. 6e,d and Supplementary Figs 1e–g,4c) or arbitrary signal intensity values for the control experiments using Fluo-3 (Fig. 7a,b).

Live-cell imaging. For cell tracking, cells were plated in 96-well plates and automated microscopy was carried out using an ImageXpress 5000A (Molecular Devices), equipped with a temperature control unit, ×4 Fluor and ×20 Plan Fluor objectives (Nikon) and a 300 W xenon arc lamp. Cell nuclei were labelled using 100 μg ml⁻¹ Hoechst 33342 for 1 h at 37 °C. As imaging medium, EGM2 supplemented with 20 μM HEPES and 1 μM of l-ascorbic acid was used. Plates were sealed and images captured every 15–30 min for 4–12 h. To measure local Ca²⁺ signals and DAG signals, cells were imaged using a custom-assembled spinning disc confocal microscope, equipped with a Zeiss 100 M microscope. The system was equipped with an automated x–y stage (ASI), and a custom-built environmental chamber (Haiso). The confocal light path was equipped with three laser, 442 nm (He–Cd, 300 mW, KIMMON), 514 nm (Ar–Kr, 400 mW, Melles Griot) and 393.5 nm (DPSS, 100 mW, CNI), a CCD (charge-coupled device) camera (CoolSNAP HQ, Photometrics) and appropriate excitation and emission filters. The epifluorescence light path was equipped with a 100 W HBO lamp, a CCD camera (CoolSNAP HQ, Photometrics) and appropriate filter sets. × 40 1.3 numerical aperture (NA) or ×63 1.4 NA Plan-APochromat objectives (Zeiss) were used and the system was controlled using μManager48. The cells were plated on the eight-well LabTek chamber slide with 40 μM HEPES, 1 μM l-ascorbic acid and 1 μM probenecid added to the EGM2 medium. Images were taken by 2 x 2 binning at 37 °C every 20 s for 6–15 min depending on the specific experimental conditions. Images of pixel size 2.5 × 2.5 μm at a speed of 0.5 x 0.5 s were acquired every 4, 20 or 40 s on images taken using FluorXpress or Nipkow microscope.

Image processing. All images were processed using Matlab 2010b (MathWorks) as described previously52. In brief, local background subtraction was applied to every image as (new value of each pixel) = (old value of each pixel) − (the median value for punctate analysis) or (the fifth percentile value (for other analysis) of the pixel’s closest neighbors of that pixel) (10–50 pixels). Borders of each cell were determined by the modified Otsu method53 for thresholds using the signals from the membrane markers and Hoechst, respectively. For STIM1 and ER images as shown in Fig. 4a and Supplementary Fig. 4a, and for FRET images using TIER as shown in Fig. 5e
and Supplementary Fig. 4f, the mask of ER from a single image was determined by summation of the YFP/FRET and CFP image. The CAAX image as shown in Supplementary Fig. 4e was not used to generate the mask because ER signals were not present within 5 μm of the cell boundary. Cell tracking was conducted by searching the nearest neighbours surrounding the specific nucleus. Specifically, the speed of sheet migration was determined by the advancement of the boundaries or the nuclei of leader cells in the front of the sheets. The parameters of individual migrating cells (speed, persistence, directionality and so on) were then calculated on the basis of the migration track of each cell. To determine the temporal changes of GFP–paxillin signal intensities after drug treatments (Fig. 2h and Supplementary Fig. 2h), a 10 μm × 10 μm square box was chosen at the leading edge of migrating cells. The average signal intensity of each time was recorded accordingly as described previously. To quantify the gradients of STIM1 (Fig. 4b,c,e and Fig. 5b–d and Supplementary Fig. 4d), cytosolic or ER luminal Ca^{2+} (Figs 1f, 4h and 6a), DAG (Fig. 1f), PtdIns(3,4,5)P_2 (Fig. 1d) and PtdIns(4,5)P_2 (Supplementary Fig. 1b–d), a ring covering the outer 20% of the area of the specific mask (ER or PM where applicable) was used to calculate the ratio in the front (0°), the back (180°) and the sides (between 0° and 180°). The local Ca^{2+} fluctuations shown in Fig. 1h and Supplementary Fig. 1h were calculated by averaging the absolute difference between local Ca^{2+} peaks and the median cytosolic level of each cell, followed by averaging the results from all cells. To compare the signal differences of phosphotyrosine (Fig. 1a,b) or Ca^{2+} (Figs 6, 7 and Supplementary Figs 5 and 6) in an individual cell, the front and back signals were calculated by averaging the signal intensities at the front 10% and the back 10% of the specific mask (ER or PM where applicable) along the direction of sheet migration.

Annotated data analysis codes are attached as the Supplementary Note, which includes Supplementary Code 1 (basic processing), Supplementary Code 2 (gradient measurement) and Supplementary Code 3 (nuclear tracking).

**Statistical tests.** Multisite imaging was done by either an automated microscope for images using ×4 or ×20 objectives, or a Nipkow spinning disc confocal microscope for images using ×40 or ×63 objectives. For sheet migration assays, three or four duplicates were carried out for each condition depending on available wells and time-lapse routines in the 96-well plates. For imaging using the Nipkow microscope, eight independent areas were taken over each of the two to four wells per condition. Therefore, although the sample sizes were not estimated before the experiments, they were generally larger than needed for adequate statistical results. For images from the ImageXpress or Nipkow microscope, every live cell at the border of the wound was used for quantitative analysis of fluorescent signals or migration parameters throughout the experiments. (To avoid too many traces in Fig. 8c, 50 cells were randomly selected from each group for demonstration, using the rand() function in Matlab. All cells were used for quantitative analysis as shown in Fig. 8c.) All bars in the figures are ±s.e.m. All statistical tests were carried out using Matlab (MathWorks), as described previously. In brief, Student’s t-test was used to compare the difference between two groups whereas one-way ANOVA was used for three or more groups. The statistical tests were chosen on the basis of the assumption that the values were normally distributed, which was validated as shown in Supplementary Fig. 7d. In addition, on the basis of the central limit theorem, the mean values of samples will approximate a normal distribution with the increase of sample size, justifying our choice of statistical tests. Specific P-values are provided in the figure panels and P < 0.05 was considered statistically significant.

**Repeatability of experiments.** In main and supplementary figures, representative images were presented as Figs 1a,c,e,g,i, 2a,h, 3c, e, 4a, 5a,c and 6d,e. Each representative image is complemented by data quantification with biological repeats, the number of which is mentioned in the figure legends. Throughout the paper, experiment were generally repeated at least three times, except those repeated twice, including Figs 2b,g, 3d,f,g, 5f,i, 6a, 7d,e,f,h, 8b,e,f, Supplementary Figs 3, 5b,d, 6a–c and 7a.

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Supplementary Figure 1  Local Ca\textsuperscript{2+} pulses in the front of migrating cells are generated via polarized receptor tyrosine kinase (RTK) signaling.  (a) Quantification of the differential signal intensities of phospho-tyrosine between the front and the back (front - back) of migrating cells (n = 107, 105, 115, 110 and 107 cells for SFM, follower cells, and Ponatinib 0 nM, 25 nM and 100 nM, respectively). SFM: The migration assay was performed in serum-free medium. FGF: The migration assay was performed in the presence of fibroblast growth factor 20 ng/mL. Bars denote mean ± SEM. (b) Negative control for Fig. 1c-f. The distribution of mCD4-YFP did not show a front-back gradient when normalized by mCD4-CFP both in leader and follower cells. (n = 64 and 212 cells for leader and follower cells, respectively). (c) The PI(4,5)P\textsubscript{2} sensor PLC\textgreek{d} -PH-YFP did not show a significant front-back gradient in migrating cells (n = 25 and 81 cells for leader and follower cells, respectively). (d) Localization gradients quantified as ratio between front and back localization. Bars denote mean ± SEM (for leader and follower cells, n = 64 and 212 cells for CD4, 24 and 42 cells for Akt-PH, 25 and 81 cells for PLCa-PH and 28 and 62 cells for PKC-C1). (e,f) [Ca\textsuperscript{2+}] levels and pulse activities increased after adding 10% fetal bovine serum and decreased after addition of the pan-RTK inhibitor Ponatinib 100 nM. Individual frames of a time-lapse sequence are shown. (g) Calibration of cytosolic [Ca\textsuperscript{2+}] using Fura-2, as described in Methods. FR denotes (F – Fmin) / (Fmax - F), where F is the Fura 340/380 ratio, Fmin is the ratio at zero [Ca\textsuperscript{2+}] and Fmax is the ratio at saturating [Ca\textsuperscript{2+}] (39 mM). (h) Similar experiment as shown in Fig. 1h and Supplementary Fig. 1e,f, but showing the effect of Ponatinib inhibition on local Ca\textsuperscript{2+} pulses in the front of FGF rather than of serum stimulated cells (middle). Ponatinib (100 nM) reversed the effect of FGF stimulation (n = 25 cells). FGF could not trigger local Ca\textsuperscript{2+} pulses when cells were pretreated with Ponatinib (100 nM for 6 minutes) (right) (n = 31 cells). The amplitude of Ca\textsuperscript{2+} fluctuation was normalized using average cell wide basal cytosolic levels (0.3 R.U. means the fluctuation was 30% of the average cytosolic [Ca\textsuperscript{2+}] level). Bars denote mean ± SEM. Student t test was used for Supplementary Fig. 1a,h.
Supplementary Figure 2 Store-operated Ca\textsuperscript{2+} (SOC) influx controls cell migration by regulating cell-matrix adhesion in the front of migrating cells. (a) Knockdown efficiency of siPMCA and siSTIM1 shown by Western blot. (b) siSTIM1, siSTIM2, siORAI1 and siORAI2 all accelerated sheet migration of HUVEC. Bars of siSTIM1, siSTIM2 and siCtrl are also shown in Fig. 3a. Student t test was used to compare siCtrl with siSTIM1, siSTIM2, siORAI1 or siORAI2. (p = 0.0199, 0.1587, 0.0506 and 0.0610, respectively.) (c) Over-expression of YFP-STIM1 rescued the effect of STIM1 knockdown on migration speed in HUVEC cells. (d) The SOC inhibitor BTP2 rescued the inhibitory effect of STIM1 over-expression on cell migration. (e) Ca\textsuperscript{2+} buffering by Fura-2 increased the speed HUVEC migration. The control compound Mag Fura-2 had no significant effect. Bars in (b) through (e) are mean ± SEM (n = 4 experiments for each group). (f) Example showing that BTP2 alters local Ca\textsuperscript{2+} signals in the front of migrating cells. The right pseudocolor graph shows time-lapse changes (x-axis) of local Ca\textsuperscript{2+} signals along the leading edge (y-axis) of the cell shown in the left panel. Both basal Ca\textsuperscript{2+} levels and the amplitudes of Ca\textsuperscript{2+} pulses decreased after BTP2 treatment. [Ca\textsuperscript{2+}] was normalized using average cytosolic levels before BTP2 treatment. (g) Quantification of the amplitudes of Ca\textsuperscript{2+} signals relative to the basal level before BTP2 treatment. The amplitude of Ca\textsuperscript{2+} fluctuation was normalized using average cell-wide basal cytosolic levels as a reference. Bars are mean ± SEM. (n = 20 cells) (h) Examples of migrating HUVEC expressing GFP-paxillin after Blebbistatin treatment. Control and BTP2-treated cells (same as Fig. 2h,i) were included for comparison. Notice the immediate decrease of the GFP-paxillin after Blebbistatin addition and the slightly delayed response after BTP2 addition. (i) Time course of loss in GFP-paxillin puncta in front following addition of the SOC inhibitor BTP2, the myosin inhibitor Blebbistatin or control (medium). Note that Blebbistatin decreased paxillin signals initially faster than BTP2. Bars are mean ± SEM (n = 14 cells for control cells, n = 36 cells for BTP2 treated cells, n = 20 cells for Blebbistatin treated cells). One-way ANOVA was used for Supplementary Fig. 2e to compare groups treated with different concentrations of (Calcium) Fura-2 or Mag Fura-2.
Supplementary Figure 3 Fibronectin and SOC regulate focal adhesions. H1299 cells were plated on different concentrations of fibronectin and treated with BTP2 or transfected with mCherry-STIM1 before fixation and staining with anti-paxillin antibody to label endogenous nascent focal adhesion complexes. (a) A separate experiment from that shown in Fig. 3d also shows more punctate paxillin signals in cells on high compared to low fibronectin. Bars are mean ± SEM (n = 47 cells on high fibronectin and 51 cells on low fibronectin group). (b) BTP2 decreases punctate paxillin signals in cells grown on high or low fibronectin. Data are analyzed from the same experiment as shown in Fig. 3d. Averaged punctate paxillin signals were compared between different cell treatments. Bars are mean ± SEM (n = 115, 121, 104 and 133 cells from left to right for each group). (c-f) H1299 cells with over-expressing mCherry-STIM1 have more punctate paxillin signals, compared to control cells expressing tdimer2-CAAX. Data were analyzed from the same experiment shown in Fig. 3f. (c,d) Correlation between the expression level of over-expressed proteins and punctate paxillin signals. Solid lines denote mean while dotted lines denote SEM; n = 89 (red) & 62 (blue) cells in (c), and 108 (red) & 70 (blue) cells in (d). (e,f) Integrated punctate paxillin signals from each cell were binned and averaged according to the level of STIM1 overexpression. N: logeRFP < 1.5. H: logeRFP > 4. Bars are mean ± SEM (n = 15, 17, 45 & 16 cells from left to right for each group in (e), and 17, 18, 42 & 23 cells from left to right for each group in (f)). (g) Full Western blot scans of Supplementary Fig. 2a, with molecular weight (MW) size markers. Student t test was used for Supplementary Fig. 3a,b,e,f.
**Supplementary Figure 4** STIM1 is enriched at the front of migrating cells to generate polarized SOC influx. (a) The ratio-image of STIM1 / ER in the HUVEC cell shown in Fig. 4a indicates an enrichment of STIM1 at the front. (b) Assay to test whether mutant STIM1 constructs regulate SOC equally well as wild-type STIM1. (c) The STIM1 mutant S1NN deficient in binding microtubule plus ends induced store-operated Ca$^{2+}$ (SOC) influx similarly as the wild-type STIM1 protein. Bars are mean ± SEM (n = 10,000 for each of the wild-type or NN mutant group). (d) Quantification of the signal ratios for STIM1 / ER-PM puncta of the cell as shown in Fig. 5a. (e,f) Images of the example shown in Fig. 5e. (e) Timelapse images of a cell expressing mCherry-CAAX as membrane marker and the ER Ca$^{2+}$ probe T1ER, migrating in the direction indicated by the arrow. (f) Original images of FRET and CFP signals. Dotted lines reveal the boundary of the cell based on the plasma membrane marker. Notice that the density of ER was very low within about 5 μm of the cell border, consistent with our previous data, and the notion that local Ca$^{2+}$ pulses ~6.6 μm behind the cell front induce lamellipodia retraction and to enhance focal adhesion. (g-i) Treatment of cells with Ponatinib reduced the activation of STIM1 in the front of migrating cells. (g) Another example showing the increase in STIM1 punctate relative to ER-PM markers in the front compared to the back of a migrating cell. The white arrow indicates the direction of cell migration. (h) Quantification of STIM1 / ER-PM ratios along the axis front-back in the cell shown in (g), before and after the addition of Ponatinib (100 nM). The white arrow indicates the reduction of relative STIM1 activation in the front. (i) The boxplot shows the median reduction of STIM1 / ER-PM gradient after addition of Ponatinib. Each box with whiskers shows, from bottom to top, minima, 25th percentile, median, 75th percentile and maxima values. Paired t test was used to generate the p value. (n = 3 cells)
Supplementary Figure 5 Higher Ca$^{2+}$ pump activity in the front generates a gradient of basal cytosolic [Ca$^{2+}$] in migrating HUVEC. (a,b) The amplitude of local lamellipodia protrusion and retraction cycles only correlated with Ca$^{2+}$ pulses when front cytosolic Ca$^{2+}$ levels were low. (a) Addition of the SERCA inhibitor thapsigargin to migrating HUVEC showed that the correlation between local Ca$^{2+}$ and front retraction was decreased when cytosolic Ca$^{2+}$ was elevated. HUVEC were loaded with Fura-2/AM as described in Methods. [Ca$^{2+}$] was normalized to average cytosolic levels before thapsigargin treatment. (b) Average changes of the front membrane speed with local Ca$^{2+}$ levels before and after thapsigargin addition. Bars are mean ± SEM (n = 6 cells). (c,d) Thapsigargin increased the Ca$^{2+}$ gradient in migrating cells, indicating that SERCA does not contribute to generate the Ca$^{2+}$ gradient. (c) Cells were plated and loaded with Fura-2/AM and imaged for 3 minutes to record baseline Ca$^{2+}$ levels before addition of thapsigargin (2 μM). Cells were then imaged for 10 more minutes. (d) Average change in cytosolic Ca$^{2+}$ levels in front versus back. Notice the differential decrease of cytosolic Ca$^{2+}$ levels in the front (left) and the back (middle) of migrating cells. The Ca$^{2+}$ gradient (back – front, right) was increased. Bars are mean ± SEM (n = 32 cells). (e,f) The PMCA inhibitors Caloxin 2A1 and La$^{3+}$ both suppressed the Ca$^{2+}$ gradient and the differential Ca$^{2+}$ pump activities in migrating cells. Bars are mean ± SEM. (e) Both PMCA inhibitors increased cytosolic Ca$^{2+}$ levels in the front and in the back (n = 47 cells for each group). At the same time, the Ca$^{2+}$ gradient ([Ca$^{2+}$]back – [Ca$^{2+}$]front) was decreased (Fig. 6c). (f) Cells pre-incubated with PMCA inhibitors were treated with thapsigargin, EGTA, and BTP2 as in Fig. 6d. This resulted in a decreased Ca$^{2+}$ pump activity in the front (left) but not in the back (right). Notice that the effect of La$^{3+}$ on the front pumping activity was weaker than that of Caloxin 2A1, likely because the Ca$^{2+}$ chelator EGTA also partially chelated La$^{3+}$ and weakened the La$^{3+}$ inhibitory effect on PMCA. Bars are mean ± SEM (n = 47 cells for each group.) Student t test was used for Supplementary Fig. 5b,e,f.
Supplementary Figure 6 Higher Ca²⁺ pump activity in the front compared to the back generates a gradient of basal Ca²⁺ in migrating cells. (a,b) 10 μM of 2,4-DCB or 3,4-DCB slightly decreases Ca²⁺ pump activities in the front and in the back of migrating HUVECs. However, the differential pump activities were not affected (Fig. 7f). La³⁺ was used as a positive control. Bars are mean ± SEM (n = 41, 40, 35 & 42 cells in DMSO, 2,4-DCB, 3,4-DCB and LaCl₃ group, respectively). (c) The PMCA inhibitor La³⁺ increased cytosolic Ca²⁺ and reduced HUVEC sheet migration, whereas EGTA (1mM) decreased cytosolic Ca²⁺ and enhanced sheet migration. Bars are mean ± SEM (n = 4 wells for each group). (d) PMCA is enriched in the front of migrating cells. Leader cells co-expressing GFP-PMCA4b and lyn-tdimer2 were analyzed using the sheet migration assay. Images of GFP-PMCA4b (left) and lyn-tdimer2 (the rest) of a migrating cell are shown. The pseudo-color image shows how the cell migrated in the direction indicated by the arrow. The corresponding ratio image GFP-PMCA4b/lyn-tdimer2 is shown in Fig. 7g. (e) H293T cells transfected with GFP-PMCA4b showed localization in the plasma membrane. Student t test was used for Supplementary Fig. 6a,b. One-way ANOVA was used in Supplementary Fig. 6c for comparison of dose-dependent effect of La³⁺ on sheet migration speed.
Supplementary Figure 7 Polarized phospholipase C (PLC) activity in the front of migrating cells controls migration through DAG production. (a) The PLC inhibitor U73122 slowed down HUVEC sheet migration in addition to single cell speed as shown in Fig. 8b of HUVEC. U73122 was added to the cell sheets prior to time-lapse imaging. (n = 2 experiments for each group). (b) Inhibition of DAG kinase caused a small but significant increase in single cell migration speed in control cells. DAGKI II had a stronger effect when cell density was low than when the density was high. 1X density ~ 31,250 cells/cm². Bars are mean ± SEM (n = 3 experiments for each group). (c) Addition of the PKCb inhibitor Ruboxistaurin (0, 5 and 10 µM) suppressed migration in a dose-dependent manner (the black, red and blue Bars at the leftmost column of the graph), while increasing doses of a DAG kinase inhibitor (0 to 4 µM) increased directed cell migration when PKCb activity was partially inhibited. Note that the effect of DAG kinase inhibition was clearly apparent when PKC was partially inhibited. Bars are mean ± SEM (n = 4 experiments for each group). (d) Histogram of the length distribution of leader cells (mean ± s.d. = 55.23 ± 16.82 µm, n = 115 cells). One-way ANOVA was used for Supplementary Fig. 7a,b.
Supplementary Figure 8  Schematic representation of the polarized Ca^{2+} signaling system components that orchestrate directed cell migration. (a) Receptor tyrosine kinase signaling is polarized in the front of migrating cells, resulting front-restricted generation of PIP_3. The local RTK activation also generates local IP_3-mediated Ca^{2+} pulses that regulate retraction and adhesion via activation of MLCK. The co-generated gradient in DAG controls speed and directionality by polarized activation of conventional PKCs. (b) STIM1 is enriched in the cell front and, in addition, selectively activated by lower luminal Ca^{2+} levels in the front of the ER. The resulting polarized store-operated Ca^{2+} influx in the front triggers local Ca^{2+} signals of its own and restores luminal Ca^{2+}, enabling local IP_3-gated Ca^{2+} release pulses to be continuously triggered. (c) Ca^{2+} extrusion by plasma membrane Ca^{2+}-ATP pumps is higher in the cell front, causing lower basal Ca^{2+} levels in the front, enabling local Ca^{2+} signals to regulate MLCK.
Supplementary Table Legends

**Supplementary Table 1:** Estimation of absolute cytosolic \([\text{Ca}^{2+}]\) values by Fura-2. A calibration curve was generated as describe in Materials and Methods and shown as Supplementary Fig. 1g using the equation \([\text{Ca}^{2+}] = \text{Kd} \times (F - F_{\text{min}})/(F_{\text{max}} - F)\) with \(F\) as the Fura 340/380 ratio. The estimated Kd of Fura-2 from our calibration curve was 101 nM, lower than the values reported by the manufacturer (145 nM) and in the literature (224 nM). However, combining the different Kd values yielded an estimate for cytosolic \([\text{Ca}^{2+}]\) of 45-105 nM, which is in agreement with literature reports.

**Supplementary Table 2:** Sequences of PCR primers used to generate diced siRNA. As described in Materials and Methods, for each gene, two diced pools of siRNAs were made to target two independent regions of gene transcript. For each siRNA pool, the targeting region was first amplified from a cDNA library by two nested PCR reactions, followed by in vitro transcription and in vitro dicing (Reference). The PCR primers used for the first and second nested PCR reactions are found in sheets FCT-ST-GSP-F, FCt-ST-GSP-R and FCt-SP-T7P-F and FCT-SP-T7P-R, respectively.

**Supplementary Note:** Matlab codes are attached, including those for image processing (Supplementary Code 1), gradient measurement (Supplementary Code 2) and nuclear tracking (Supplementary Code 3). Instructions at the beginning of each file describe how to generate codes and to run image & quantification analysis. Further assistance for using the code may be available on request by F.C.T.

**Supplementary Movie Legend**

**Supplementary Movie 1.** The frequency of local \([\text{Ca}^{2+}]\) pulses is higher in the front than in the middle or the back of migrating leader cells during collective migration. The movie shows local \([\text{Ca}^{2+}]\) activities in leader cells. GCaMP6 tethered to the PM using a CAAX sequence was used to monitor temporal and spatial \([\text{Ca}^{2+}]\) changes. Three of the nine traces are also shown as a static image in Fig. 1g.
Supplementary Code 1: Basic processing.
This file contains the functions required by the matlab scripts for data analysis. The users should copy the text from each function and save it as separate .m files with filenames specified as below, together with other scripts for data analysis.

Filename: bgsub.m
function ImageNew=bgsub(ImageOld,Radius,fra)
% fra: fraction; if zero, will be generated automatically

%% flatten the image
ImageNew=ImageOld-getbg_simple(ImageOld,Radius,fra);

Filename: getbg_simple.m
function bg=getbg_simple(img,rd,bgpeak)
% bgpeak: fraction (0-1); if zero, will be generated automatically

%% calculate the ratio
ratio=min([1,20/rd]);
rd_new=min([rd,20]);
mkbg=getnhood(strel('disk',rd_new,0));szb=size(mkbg,1);
mksm=getnhood(strel('disk',round(rd_new/2),0));szs=size(mksm,1);
x1=1+(szb-szs)/2;x2=x1+szs-1;
mk=mkbg;mk(x1:x2,x1:x2)=mkbg(x1:x2,x1:x2)-mksm;

%% calculate bg peak
if bgpeak==0
    bgpeak=(4-pi)/8;
end

%% use ordfilt2 to get bg
im_small=imresize(img,ratio,'bicubic');
bg_small=ordfilt2(im_small,ceil(sum(mk(:))*bgpeak),mk,'symmetric');
bg0=imresize(bg_small,size(img),'bicubic');
bg=imfilter(bg0,fspecial('disk',min([rd,32])),'replicate');
Filename: CalcJitter.m

function [xx,yy,minV]=CalcJitter(DapiImage0,DapiImage)
% DapiImage0:original image, DapiImage:new image

%% Get mask
%Mask0=GetM(DapiImage0);Mask=GetM(DapiImage);
Mask0=DapiImage0;Mask=DapiImage;

%% Calculation
xcoor=0;ycoor=0;
[sy,sx]=size(DapiImage);

r0=3.*(0:3);r0=r0(r0<=ceil(min([sx,sy])/6));

for r=fliplr(r0);
    Mask0f=imfilter(Mask0,fspecial('disk',r),'symmetric');
    Maskf=imfilter(Mask,fspecial('disk',r),'symmetric');
    x0=xcoor+1;y0=ycoor+1;%make while loop running
    while ((xcoor~=x0)||(ycoor~=y0))&&((abs(xcoor)<sx)&(abs(ycoor)<sy))
        x0=xcoor;y0=ycoor;
        [xcoor,ycoor,TempStatM,minV]=JitterCalc(Mask0f,Maskf,x0,y0,r);
    end
end

%% get better value
px=zeros(1,3);py=zeros(1,3);
ts=TempStatM;
for rd=1:3
    ts2=imresize(ts,[61,61],'bicubic');
    ts3=ts2; ts3(:,:,1:3)=[]; ts3(:,:,end-2:end)=[]; ts3(1:3,:)=[]; ts3(end-2:end,:)=[]; %take out the outer
3 rows and 3 columns
    [~,tpx]=min(min(ts3,[],1));
    [~,tpy]=min(min(ts3,[],2));
    tpx=tpx+3; tpy=tpy+3; px(1,rd)=(tpx-31)/(10^rd);
    py(1,rd)=(tpy-31)/(10^rd);
    ts=ts2((tpy-3):(tpy+3),(tpx-3):(tpx+3));
end
xx=xcoor+sum(px);
yy=ycoor+sum(py);

% TempStatM2=TempStatM(3:5,3:5);
% TempStatM3=imresize(TempStatM2,[201,201],'bicubic');
% [~,px]=min(min(TempStatM3,[],1));
% [~,py]=min(min(TempStatM3,[],2));
% TempStatM4=imresize(TempStatM3(py-1:py+1,px-1:px+1),[201,201],'bicubic');
% [~,px2]=min(min(TempStatM4,[],1));
% [~,py2]=min(min(TempStatM4,[],2));
% xx=xcoor+(px-101)/100+(px2-101)/10000;
% yy=ycoor+(py-101)/100+(py2-101)/10000;

Filename: JitterCalc.m

function [xcoor,ycoor,TempStatM,minV]=JitterCalc(ImageO,ImageN,x0,y0,r)

%ImageO:original,ImageN:new,[x0,y0]:starting coor,r:grid
%xcoor,ycoor=resulting coor
%ImageN=YfpImage;ImageO=YfpImage;r=4;x0=4;y0=4;

%%% preparation
ymatrix=y0+(-3:3)'*ones(1,7)*r;xmatrix=x0+ones(7,1)*(-3:3)*r;

%%% calculation
TempStat=zeros(49,3);
TempStatM=zeros(7,7);
for rows=1:7
    for cols=1:7
        dy=ymatrix(rows,cols);dx=xmatrix(rows,cols);

        ImageN2=ImageN(max(1,1-dy):min(end,end-dy),max(1,1-dx):min(end,end-dx));
        ImageO2=ImageO(max(1,1+dy):min(end,end+dy),max(1,1+dx):min(end,end+dx));
        ImageD=abs(ImageN2-ImageO2);

        TempStat(rows*7-7+cols,:)=dx,dy,sum(ImageD(:))/size(ImageD(:),1));
        TempStatM(rows,cols)=sum(ImageD(:))/size(ImageD(:,1));
    end
end
end
end

%% sorting and get resulting coor
[minV,I]=min(TempStat(:,3));
xcoor=TempStat(I,1);ycoor=TempStat(I,2);

Filename: CropJitter
function imnew=CropJitter(imold,cx1,cx2,cy1,cy2,dx,dy)

% cx1,cx2,cy1,cy2: margin
% dx,dy: jitter

%% get floor and remain
dxf=floor(dx);dxr=dx-dxf;
dyf=floor(dy);dyr=dy-dyf;

%% get crude image
new11=imold(1+cy1 -dyf:end-cy2-dyf,1+cx1-dxf:end-cx2-dxf);
new12=imold(cy1-dyf:end-cy2-dyf-1,1+cx1-dxf:end-cx2-dxf);
new21=imold(1+cy1 -dyf:end-cy2-dyf,cx1-dxf:end-cx2-dxf-1);
new22=imold(cy1-dyf:end-cy2-dyf-1,cx1-dxf:end-cx2-dxf-1);

%% intrapolation
new1y=(1-dyr)*new11+dyr*new12;
new2y=(1-dyr)*new21+dyr*new22;
imnew=(1-dxr)*new1y+dxr*new2y;

Filename: getdapimask
function da_ma=getdapimask(da_bs,nucr)

%% threshold mask
da_bs_g=mat2gray(da_bs);
th_gray=graythresh(da_bs_g);
th_high=min(da_bs(da_bs_g>th_gray));
% [~,th_high] = ThreshImage(da_bs);
[~,th_low] = ThreshImage(da_bs);

da_mas = (da_bs > 1/mean([1/th_high, 1/th_low]));

%%% edge mask

% da_bb = [da_bs(:, 1+nucr:end-1:-1), da_bs, da_bs(:, end-1:-1:end-nucr)];
da_bb = [da_bb(1+nucr:end-1:-1:end-nucr,:), da_bb, da_bb(:, end-1:-1:end-nucr,:)];

% da_bdx = 2*da_bb(1+nucr:end-nucr, 1+nucr:end-nucr) - da_bb(1:end-2*nucr,1+nucr:end-nucr) - da_bb(1+2*nucr:end,1+nucr:end-nucr);
da_bdy = 2*da_bb(1+nucr:end-nucr, 1+nucr:end-nucr) - da_bb(1:end-2*nucr,1+2*nucr:end) - da_bb(1+2*nucr:end,1:end-2*nucr);
da_bdr = 2*da_bb(1+nucr:end-nucr, 1+nucr:end-nucr) - da_bb(1:end-2*nucr,1+2*nucr:end) - da_bb(1+2*nucr:end,1:end-2*nucr);
da_bdl = 2*da_bb(1+nucr:end-nucr, 1+nucr:end-nucr) - da_bb(1+2*nucr:end,1:end-2*nucr) - da_bb(1+2*nucr:end,1+2*nucr:end);
da_bd = da_bdy + da_bdx + da_bdr + da_bdl;

% da_bdg = mat2gray(da_bd);
da_mag0 = ThreshImage_ultrasen(da_bd);

% da_m0 = imfill((da_mas & da_mag), 'holes');

% segmentation by local maxima

da_bsd = [da_bs(:, 1+1), da_bs, da_bs(:, end-1)];
da_bsd = [da_bsd(1+1,:), da_bsd, da_bsd(end-1,:)];

da_bs0 = zeros(size(da_bs));

for i=0:2
    for j=0:2
        tempdiff = (da_bs - da_bsd(1+i:end-2+i, 1+j:end-2+j));
    end
end
da_bsf=da_bsf+(tempdiff>=0);
end
end
da_pk=(da_bsf>=8)&(da_ma0);
da_pkd=imdilate(da_pk,strel('disk',floor(nucr*2/3),0));
da_wa=watershed(bwdist(da_pkd))>0;
da_ma1=(da_ma0&da_wa);

%% segmentation of da_mas
% da_wa2=watershed(bwdist(da_ma1))>0;
% da_ma2=(da_mas&da_wa2);

%% filtering
da_ma=bwareaopen(da_ma1,round(nucr.^2/2));

Filename: ThreshImage.m
function [ImageMask,th,bg]=ThreshImage(ImageOld)
%ngps:number of groups
%cutafter:cut after which group (from left)

%% make judgement
ImageOld=single(ImageOld);
TempSeries=ImageOld(:);

%% get the histogram of the image values
tmax=max(TempSeries);
tmin=min(TempSeries);
nbin=200;tbin=(tmax-tmin)/nbin;
tmin=tmin+tbin/2;tmax=tmax-tbin/2;
[n,xout]=ksdensity(TempSeries,tmin:tbin:tmax);
gp=max([2,ceil(nbin/50)]);
ng=getcurvature(n,gp);

%% get background value
negpeak=regionprops(ng<0,'PixelIdxList','Area');
negth=prctile(ng,25)-1.5*iqr(ng);
Ibg00=zeros(1,size(negpeak,1));AC=Ibg00;DD=Ibg00;ACsum=Ibg00;
for cc=1:size(negpeak,1)
    AC(cc)=negpeak(cc).Area;
    tempeakvalues=ng(negpeak(cc).PixelIdxList);
    ACsum(cc)=sum(log(-tempeakvalues+1));
    [DD(cc),I]=min(tempeakvalues);
    Ibg00(cc)=negpeak(cc).PixelIdxList(I);
end
neglo=((DD<=negth)|(AC>=gp));Ibg00=Ibg00(neglo);DD=DD(neglo);ACsum=ACsum(neglo);
if isempty(Ibg00)
    [~,Ibg00]=min(ng);
end
Ibg00=[Ibg00,size(ng,2)-gp];ACsum=[ACsum,0];
[~,I_DD]=min(DD);
Ibg0=Ibg00(I_DD);
bg0=xout(Ibg0);%approximate value
TempSeries2=TempSeries((TempSeries>(bg0-5*gp*tbin))&(TempSeries<(bg0+5*gp*tbin)));
[n_bg,xout_bg]=ksdensity(TempSeries2);
[~,Ibg]=max(n_bg);%better value
if Ibg==1;Ibg=2;elseif Ibg==100;Ibg=99;end
xfit=xout_bg(Ibg-1:Ibg+1);yfit=n_bg(Ibg-1:Ibg+1);
%det_n=det([xfit.^2,xfit,[1;1;1]]);%/det_n;
a=det([yfit,xfit,[1;1;1]]);%/det_n;
b=det([xfit.^2,yfit,[1;1;1]]);%/det_n;
bg=-b/a;2;%best value

%%% get upperbound
Ibg01=Ibg00;Ibg01(xout(Ibg00)<bg)=[];
%DD1=DD;DD1(1:I_DD)=[];
AC1=ACsum;AC1(xout(Ibg00)<bg)=[];
[~,I_DD1]=max(AC1);
Ibg1=Ibg01(I_DD1);
upperb=min([10*(xout(Ibg1)-bg)+bg,xout(end)]);
%upperb=(xout(Ibg00(I_DD+1))+10*gp*tbin);

%%% get threshold
TempSeries3=TempSeries((TempSeries>(bg-gp*tbin))&(TempSeries<upperb));
[n_th,xout_th]=ksdensity(TempSeries3,bg:(upperb-bg)/100:upperb);
[~,Ibg]=min(abs(xout_th-bg));
ng_th = getcurvature(n_th, gp);
partng = ng_th(Ibg:end);
pospeak = regionprops(partng > 0, 'PixelIdxList', 'Centroid', 'Area');
Ith00 = zeros(1, size(pospeak, 1)); AC = Ith00;
for cc = 1:size(pospeak, 1)
    AC(cc) = sum(partng(pospeak(cc).PixelIdxList));
    %AC(cc) = pospeak(cc).Area;
    Ith00(cc) = round(pospeak(cc).Centroid(1));
    %Ith00(cc) = (sum((pospeak(cc).PixelIdxList)'.*(partng(pospeak(cc).PixelIdxList)))/AC(cc));
end
 [~, I] = max(AC); Ith0 = floor(Ith00(I)); Ithr = (Ith00(I) - Ith0);
s1 = xout_th(Ith0 + Ibg - 1); s2 = xout_th(Ith0 + Ibg);
th = s1 + Ithr*(s2 - s1);

%% get mask
ImageMask = (ImageOld > th);

Filename: ThreshImage_ultrasen.m
function [ImageMask, th, bg] = ThreshImage_ultrasen(ImageOld)
%ngps: number of groups
%cutoff: cut after which group (from left)

%% make judgement
ImageOld = single(ImageOld);
TempSeries = ImageOld(:);

%% get the histogram of the image values
tmax = max(TempSeries);
tmin = min(TempSeries);
nbin = 200; tbin = (tmax - tmin)/nbin;
tmin = tmin + tbin/2; tmax = tmax - tbin/2;
[n, xout] = ksdensity(TempSeries, tmin: tbin: tmax);
gp = max([2, ceil(nbin/50)]);
ng = getcurvature(n, gp);

%% get background value
negpeak = regionprops(ng < 0, 'PixelIdxList', 'Area');
negth=prctile(ng,25)-1.5*iqr(ng);
Lbg00=zeros(1,size(negpeak,1));AC=Lbg00;DD=Lbg00;
for cc=1:size(negpeak,1)
    AC(cc)=negpeak(cc).Area;
    tempeakvalues=ng(negpeak(cc).PixelIdxList);
    [DD(cc),I]=min(tempeakvalues);
    Lbg00(cc)=negpeak(cc).PixelIdxList(I);
end
neglo=((DD<=negth)|(AC>=gp));Ibg00=Lbg00(neglo);DD=DD(neglo);
if isempty(Ibg00)
    [~,Ibg00]=min(ng);
end
Ibg00=[Ibg00,size(ng,2)-gp];
[~,I_DD]=min(DD);
Ibg0=Ibg00(I_DD);
bgo=xout(Ibg0);%approximate value
TempSeries2=TempSeries((TempSeries>(bg0-5*gp*tbin))&(TempSeries<(bg0+5*gp*tbin)));
[n_bg,xout_bg]=ksdensity(TempSeries2);
 [~,I]=max(n_bg);%better value
xfit=xout_bg(Ibg-1:Ibg+1);yfit=n_bg(Ibg-1:Ibg+1);
det_n=det([xfit.^2,xfit,[1;1;1]])
da=det([yfit,xfit,[1;1;1]])/det_n;
b=det([xfit.^2,yfit,[1;1;1]])/det_n;
bg=-b/a;%best value
%
% get threshold
TempSeries3=TempSeries((TempSeries>(bg-gp*tbin))&(TempSeries<(xout(Ibg00(I_DD+1))+gp*tbin)));
[n_th,xout_th]=ksdensity(TempSeries3,bg:(xout(Ibg00(I_DD+1))-bg)/100:xout(Ibg00(I_DD+1)));
[~,I]=min(abs(xout_th-bg));
ng_th=getcurvature(n_th,gp);
partng=ng_th(Ibg:end);
pospeak=regionprops(partng>0,'PixelIdxList','Centroid','Area');
% Ith00=zeros(1,size(pospeak,1));AC=Ith00;
% for cc=1:size(pospeak,1)
%     AC(cc)=sum(partng(pospeak(cc).PixelIdxList));
%     %AC(cc)=pospeak(cc).Area;
%     %Ith00(cc)=round(pospeak(cc).Centroid(1));
ith00(cc) = (sum((pospeak(cc).PixelIdxList).*(partng(pospeak(cc).PixelIdxList)))/AC(cc));
% end
[~,I]=max(partng(pospeak(1).PixelIdxList));
ith00=pospeak(1).PixelIdxList(I);
%[~,I]=max(AC);
%ith0=floor(ith00(I));ithr=(ith00(I)-ith0);
ith0=floor(ith00);ithr=(ith00-ith0);
s1=xout_th(ith0+ibg-1);s2=xout_th(ith0+ibg);
th=s1+ithr*(s2-s1);

%% get mask
ImageMask=(ImageOld>th);

Filename: getcurvature.m
%%
function ng=getcurvature(n,gp)

% ns=[n(1+gp:-1:1+1),n,n(end-1:-1:end-gp)];
theta1=atan2(ns(1+gp:end-gp)-ns(1:end-2*gp),gp);
theta2=atan2(ns(1+2*gp:end)-ns(1+gp:end-gp),gp);
ng_a=acos(cos(theta2-theta1));
ng_v=ns(1:end-2*gp)+ns(1+2*gp:end)-2*ns(1+gp:end-gp);
ng=ng_a.*(ng_v>0)-ng_a.*(ng_v<0);

Filename: relocateD.m
function sp=relocateD(data0)
% nchannel means the number of channels in fluorescent signals
% if yfp data is included, nchannel=1.
% if only position data is in, nchannel=0.

% data=data0;
fmin=1;fmax=size(data,3);
sp=cell(size(data));
for concon=1:size(data,1); %row
    for sese=1:size(data,2); %column
        if size(data{concon,sese,fmin},1)>0
            data{concon,sese,fmin}=unique(data0{concon,sese,fmin},'rows');
            sp{concon,sese,fmin}=data{concon,sese,fmin};
            x1=data{concon,sese,fmin}(:,1);y1=data{concon,sese,fmin}(:,2);
            for f=fmin+1:fmax
                data{concon,sese,f}=unique(data0{concon,sese,f},'rows');
                sp{concon,sese,f}=zeros(size(sp{concon,sese,f-1}));
                % preparation
                slo=size(x1,1);nlo=(x1>0);
                ser=(1:slo);ser=ser(nlo);
                x1=x1(nlo);x2=data{concon,sese,f}(:,1);X=double([x1;x2]);
                y1=y1(nlo);y2=data{concon,sese,f}(:,2);Y=double([y1;y2]);
                %z1=zeros(size(x1,1),1);z2=ones(size(x2,1),1);Z=[z1;z2];
                status12=zeros(size(x1,1),1);status21=zeros(size(x2,1),1);
                MMM=max(x1)-min(x1);
                dist12=ones(size(x1,1),1)*MMM;dist21=ones(size(x2,1),1)*MMM;
                neig12=cell(size(x1,1),1);neig12d=cell(size(x1,1),1);
                %% identifying static cells
                [NEWXY,ind]=sortrows([X,Y]);
                NEWXY_G=sum((NEWXY(2:end,:) -NEWXY(1:(end-1),:)).^2,2);
                NEWXY_lo=(NEWXY_G==0);NEWXY_lo1=logical([NEWXY_lo;0]);NEW XY_lo2=logical([0;NEWXY_lo]);
                stat1=ind(NEWXY_lo1);stat2=ind(NEWXY_lo2);
                status12(stat1)=stat2-size(x1,1);dist12(stat1)=0;
                status21(stat2-size(x2,1))=stat1;dist21(stat2-size(x2,1))=0;
                oldlabel=(1:size(X,1))';statlabel=[stat1;stat2];
                X(statlabel)=[ ];Y(statlabel)=[ ];oldlabel(statlabel)=[ ];
            end
        end
    end
end
td = delaunay(X,Y); td_oldlabel = oldlabel(td);
tdx = X(td); tdy = Y(td);
tdl = single(td_oldlabel > size(x1,1));

if size(tdx,2) == 1
    tdx = tdx'; tdy = tdy';
end

X1 = [tdx(:,1),tdx(:,1),tdx(:,2)];
Y1 = [tdy(:,1),tdy(:,1),tdy(:,2)];
LO1 = [tdl(:,1),tdl(:,1),tdl(:,2)];

X2 = [tdx(:,2),tdx(:,3),tdx(:,3)];
Y2 = [tdy(:,2),tdy(:,3),tdy(:,3)];
LO2 = [tdl(:,2),tdl(:,3),tdl(:,3)];

DIS = sqrt((X2-X1).^2 + (Y2-Y1).^2);
LO = abs(LO2 - LO1);

DISA = sort(DIS,2);
dis = median(DISA(:,1));

% startp = [1,1,2,2,2,3];
% endp = [2,3,4,3,4,4];
startp = [1,1,2];
endp = [2,3,3];

for i = 1:size(td,1)
    temp_pos = td_oldlabel(i,:);
    temp_dis = DIS(i,:);
    temp_log = LO(i,:);
    for j = 1:3
        if temp_log(j) > 0
            tempp = temp_pos([startp(j), endp(j)]);
            tp1 = min(tempp);
            tp2 = max(tempp) - size(x1,1);
            neig12{tp1} = [neig12{tp1}, tp2];
            neig12d{tp1} = [neig12d{tp1}, temp_dis(j)];
            if temp_dis(j) < dist12(tp1)
                dist12(tp1) = temp_dis(j);
                status12(tp1) = tp2;
            end
            if temp_dis(j) < dist21(tp2)
                dist21(tp2) = temp_dis(j);
                status21(tp2) = tp1;
            end
        end
    end
end

%% pairing

pair12 = zeros(size(x1,1),1);
pair21 = zeros(size(x2,1),1);
nx2=zeros(size(x1,1),1); ny2=zeros(size(x1,1),1);
for i=1:size(x2,1)
    if (status21(i)>0) && (status12(status21(i))==i)
        sp{concon,sese,f}(ser(status21(i)),:)=data{concon,sese,f}(i,:);
        %nx2(status21(i))=x2(i); ny2(status21(i))=y2(i);
        pair21(i)=1; pair12(status21(i))=1;
    end
end

%% check remaining
for i=(find(pair12==0))'
    [temp_nei,m00]=unique(neig12{i});
    temp_dis=neig12d{i}(m00);
    temp_log=(pair21(temp_nei)==0)';
    if sum(temp_log)>0
        temp_nei=temp_nei(temp_log);
        temp_dis=temp_dis(temp_log);
        [B,IX]=min(temp_dis);
        if B<dis
            %nx2(i)=x2(temp_nei(IX)); ny2(i)=y2(temp_nei(IX));
            sp{concon,sese,f}(ser(i,:),:)=data{concon,sese,f}(temp_nei(IX),:);
            pair12(i)=1; pair21(temp_nei(IX))=1;
        end
    end
end

%nx2=[nx2;x2(~pair21)]; ny2=[ny2;y2(~pair21)];
sp{concon,sese,f}=[sp{concon,sese,f};data{concon,sese,f}(~pair21,:)];

%% wrapping up
x1=sp{concon,sese,f}(:,1); y1=sp{concon,sese,f}(:,2);
end
end
end

Filename: modifysp.m

function [newsp,trk_rc]=modifysp(wellsp)
%% create track logic matrix
trk_or=zeros(size(wellsp{end},1),size(wellsp,3));%tracks
siz_or=zeros(size(trk_or));%sizes
sig_or=zeros(size(trk_or));%signals
for f=1:size(wellsp,3)
    trk_or(1:size(wellsp{f},1),f)=sum(abs(wellsp{f}),2)~=0;
siz_or(1:size(wellsp{f},1),f)=wellsp{f}(:,4);
sig_or(1:size(wellsp{f},1),f)=wellsp{f}(:,3);
end

%% create track information matrix
trk_df=diff(trk_or,1,2);siz_df=diff(siz_or,1,2);sig_df=diff(sig_or,1,2);
% set up trk_rc
trk_rc=zeros(size(trk_or,1),5);
%1~5: start frame,split from,end frame,merge to,cell#
% set up siz_vr and sig_vr
siz_vr0=sum(siz_df,2)./sum(siz_or(:,1:end-1),2);siz_vr=prctile(siz_vr0(isfinite(siz_vr0)),[2.5,97.5]);
clear siz_df
sig_vr0=sum(sig_df,2)./sum(sig_or(:,1:end-1),2);sig_vr=prctile(sig_vr0(isfinite(sig_vr0)),[2.5,97.5]);
clear sig_df
% start frame
[r,c]=find(trk_df==1);c=c+1;
trk_rc(:,1)=1;trk_rc(r,1)=c;
% end frame
[r,c]=find(trk_df==-1);
clear trk_df
trk_rc(:,3)=size(trk_or,2);trk_rc(r,3)=c;
% connect short tracks
id0=1:size(trk_or,1);trk_rc(:,2)=id0;trk_rc(:,4)=id0;trk_rc(:,5)=id0;
for f=1:size(wellsp,3)-1
    % find candidates and set up distance threshold
    sid=find(trk_rc(:,1)==f+1 & trk_rc(:,3)>f+1);
smt=ppdist2(wellsp{f+1}(sid,1:2),wellsp{f}(:,1:2));
    Cs=min(smt,[],2);
eid=find(trk_rc(:,3)==f & trk_rc(:,1)<f);
    emt=ppdist2(wellsp{f}(eid,1:2),wellsp{f+1}(:,1:2));
    Ce=min(emt,[],2);
    f_c=[Cs;Ce];f_th=prctile(f_c,75)+1.5*iqr(f_c);
%% set up start point
flo=find(wellsp{f}(:,1)>0);fsz=size(flo,1);ssz=size(sid,1);
temptri=DelaunayTri([wellsp{f}(flo,1:2);wellsp{f+1}(sid,1:2)]);
newsid=fsz+(1:ssz);
for cc=1:ssz
    [r,~]=find(temptri.Triangulation==newsid(cc));
tempmtx=temptri.Triangulation(r,:);
tempidx0=unique(tempmtx);tempidx0=tempidx0(tempidx0<=fsz);
    if ~isempty(tempidx0)
        tempidx=flo(tempidx0);
        dis=smt(cc,tempidx)',%disvec=(dis<f_th);
        dismax=max(dis);
        convec=(wellsp{f+1}(tempidx,1)==0);
        %size
        siz_bs=wellsp{f}(tempidx,4);siz_as=siz_bs;
        siz_as(~convec)=wellsp{f+1}(tempidx(~convec),4);
        sizvec=((siz_as-siz_bs)./siz_bs<=siz_vr(1));
        %signal
        sig_bs=wellsp{f}(tempidx,3);sig_as=sig_bs;
        sig_as(~convec)=wellsp{f+1}(tempidx(~convec),3);
        sigvec=((sig_as-sig_bs)./sig_bs<=sig_vr(1));
        %total
        totvec=(convec+(sizvec+sigvec)/2-(dis-dismax)/f_th*10);maxvec=max(totvec);
        if maxvec>0
            cnd=find(totvec==maxvec);
            [~,I]=min(dis(cnd));
            trk_rc(sid(cc),2)=tempidx(cnd(I));
        end
    end
end
%% set up end point
flo=find(wellsp{f+1}(:,1)>0);fsz=size(flo,1);esz=size(eid,1);
temptri=DelaunayTri([wellsp{f+1}(flo,1:2);wellsp{f}(eid,1:2)]);
neweid=fsz+(1:esz);
for cc=1:esz
    [r,~]=find(temptri.Triangulation==neweid(cc));
tempmtx=temptri.Triangulation(r,:);
tempidx0=unique(tempmtx);tempidx0=tempidx0(tempidx0<=fsz);
if ~isempty(tempidx0)
    tempidx=flo(tempidx0);
    dis=emt(cc,tempidx); % disvec = (dis < f_th);
    dismax=max(dis);
    convec=(tempidx>size(wellsp{f},1));
    % size
    siz_am=wellsp{f+1}(tempidx,4); siz_bm=siz_am;
    siz_bm(~convec)=wellsp{f}(tempidx(~convec),4);
    sizvec=((siz_am-siz_bm)/siz_bm>=siz_vr(2));
    % signal
    sig_am=wellsp{f+1}(tempidx,3); sig_bm=sig_am;
    sig_bm(~convec)=wellsp{f}(tempidx(~convec),3);
    sigvec=((sig_am-sig_bm)/sig_bm>=sig_vr(2));
    % total
    totvec=(convec+(sizvec+sigvec)/2 -(dis-dismax)/f_th*10); maxvec=max(totvec);
    if maxvec>0
        cnd=find(totvec==maxvec);
        [~,I]=min(dis(cnd));
        trk_rc(eid(cc),4)=tempidx(cnd(I));
    end
end

%% start repairing
%trk_rc0=trk_rc;
orphanlo=zeros(size(trk_rc,1),1);
newsp=wellsp;clear wellsp

%% remove single spots
lo=(trk_rc(:,1)==trk_rc(:,3));
orphanlo(lo)=1;

%% detect missing connection
trk_en=trk_rc(:,[3,4,5]); trk_en(:,1)=trk_en(:,1)+1;
trk_st=trk_rc(:,[1,2,5]);
conind=zeros(size(trk_rc,1),2);conlen=0;
for ff=2:size(trk_or,2)
    lo1=trk_en(:,1)==ff;lo2=trk_st(:,1)==ff;
    trk_ent=trk_en(lo1,:);trk_stt=trk_st(lo2,:);
    tempmtx=ppdist2(trk_ent(:,2),trk_stt(:,3));
    [conr1,conc1]=find(tempmtx==0);
    tempmtx=ppdist2(trk_ent(:,3),trk_stt(:,2));
    [conr2,conc2]=find(tempmtx==0);
    tempind=unique([conr1(:),conc1(:);conr2(:),conc2(:)],'rows');
    tempsz=size(tempind,1);
    if ~isempty(tempind)
        tempind(:,1)=trk_ent(tempind(:,1),3);
        tempind(:,2)=trk_stt(tempind(:,2),3);
        conind(1+conlen:tempsz+conlen,:)=tempind;
        conlen=conlen+tempsz;
    end
end
if conlen>0
    conind=conind(1:conlen,:);
    trk_con=[trk_en(conind(:,1),[1,3]),trk_st(conind(:,2),3)]; % trk_con: frame, mom, dau
    consiz=[diag(siz_or(trk_con(:,2),trk_con(:,1)-1)),diag(siz_or(trk_con(:,3),trk_con(:,1)))];
    consig=[diag(sig_or(trk_con(:,2),trk_con(:,1)-1)),diag(sig_or(trk_con(:,3),trk_con(:,1)))];
    consizd=diff(consiz,1,2)./consiz(:,1);
    consigd=diff(consig,1,2)./consig(:,1);
    consizl=(consizd>=siz_vr(1))&(consizd<=siz_vr(2));
    consigl=(consigd>=sig_vr(1))&(consigd<=sig_vr(2));
    tlo=consizl&consigl;
    trk_con=trk_con(tlo,:);consigd=consigd(tlo);consizd=consizd(tlo);
end
% check if all one-to-one correspondence
trk_label=zeros(size(trk_con,1),1);
condis1=squareform(pdist(trk_con(:,[1,2])));
[dis1row,dis1col]=find(condis1==0);dis1lo=(dis1row<dis1col);
trk_bad1=[dis1row(dis1lo),dis1col(dis1lo)];
condis2=squareform(pdist(trk_con(:,[1,3])));
[dis2row,dis2col]=find(condis2==0);dis2lo=(dis2row<dis2col);
trk_bad2=[dis2row(dis2lo),dis2col(dis2lo)];
trk_bad = [trk_bad1; trk_bad2];
if size(trk_bad,1)>0
    uni_bad = sort(unique(trk_bad(:,1))); lon_bad = sort(trk_bad(:,1));
    if length(uni_bad)<length(lon_bad)
        [lon_hist,xi] = hist(lon_bad,uni_bad); worm = xi(lon_hist>1);
        for wormc=1:size(worm,1)
            [wormr,~] = find(trk_bad==worm(wormc)); trk_bad(wormr,:) = [];
            trk_label(worm(wormc)) = 1;
        end
    end
end
for cc=1:size(trk_bad,1)
    tempsdf = abs(consigd(trk_bad(cc,:)));
    tempzdf = abs(consizd(trk_bad(cc,:)));
    tempidx = (diff([tempsdf,tempzdf],1,1)>0)-(diff([tempsdf,tempzdf],1,1)<0);
    tix = 1+(tempidx(1)*100+tempidx(2))*100;
    trk_label(trk_bad(cc,tix)) = 1;
end
end

%% repair missing connection
trk_con(trk_label==1,:) = [];
for cc=1:size(trk_con,1)
    % ori and dau are the same cell
    % link dau to ori
    x = (trk_rc(:,2)==trk_con(cc,3)); trk_rc(x,2) = trk_con(cc,2);
    x = (trk_rc(:,4)==trk_con(cc,3)); trk_rc(x,4) = trk_con(cc,2);
    trk_rc(trk_con(cc,2),[3,4]) = trk_rc(trk_con(cc,3),[3,4]);
    for f = trk_con(cc,1):trk_rc(trk_con(cc,2),3)
        newsp{f}(trk_con(cc,2),:) = newsp{f}(trk_con(cc,3),:);
    end
    % remove dau record
    orphanlo(trk_con(cc,3)) = 1;
    trk_con(trk_con(:,2)==trk_con(cc,3),2) = trk_con(cc,2);
end
end

%%
clear siz_or sig_or
%% correct merge (preparation)
mergelo=(trk_rc(:,4)~=trk_rc(:,5))&(~orphanlo);
mermtx=trk_rc(mergelo,3:5);mermtx=sortrows(mermtx,1);
mergelo=mermtx(:,3);

%% correct merge (start)
for cc=1:size(mermtx,1)
    tempori=trk_rc(mergelo(cc),[3,5]);
    tempmom=trk_rc(tempori(2),4);
    tempsplit=find((trk_rc(:,2)==tempmom)&(trk_rc(:,1)>tempori(1))&(~orphanlo));
    if (trk_rc(tempmom,1)<=tempori(1))
        %merge exists
        siz_bm=newsp{tempori(1)}(tempmom,4);siz_am=newsp{tempori(1)+1}(tempmom,4);
        sig_bm=newsp{tempori(1)}(tempmom,3);sig_am=newsp{tempori(1)+1}(tempmom,3);
        if (((siz_am-siz_bm)/siz_bm>=siz_vr(2))||((sig_am-sig_bm)/sig_bm>=sig_vr(2))
            % true merge
            splitlo=0;
            if size(tempsplit,1)>0
                % both merge and split occur
                tempdau=trk_rc(tempsplit,[1,5]);
                % take the first frame that shows split
                tempdau=sortrows(tempdau);
                for ccc=1:size(tempdau,1)
                    if (splitlo==0)
                        tempdaut=tempdau(ccc,:);
                        if (trk_rc(tempmom,3)>=tempdaut(1))
                            siz_bs=newsp{tempdaut(1)-1}(tempmom,4);siz_as=newsp{tempdaut(1)}(tempmom,4);
                            sig_bs=newsp{tempdaut(1)-1}(tempmom,3);sig_as=newsp{tempdaut(1)}(tempmom,3);
                            if (((siz_as-siz_bs)/siz_bs<=siz_vr(1))||((sig_as-sig_bs)/sig_bs<=sig_vr(1))
                                % true split
                                splitlo=1;
                                tempdau=tempdaut;
                            end
                        end
                    end
                end
            end
        end
    end
end
if (splitlo==1)
    % true split
    % fill the gap between ori and dau
    for f=tempori(1)+1:tempdau(1)-1
        newsp{f}(tempori(2),:)=newsp{f}(tempmom,:);
    end
    % fix tracks after split
    trkmntx=trk_rc([tempori(2),tempmom,tempdau(2)],:);
    orisig0=newsp{tempori(1)}([tempori(2),tempmom],[3,4]);
    dausig0=newsp{tempdau(1)}([tempdau(2),tempmom],[3,4]);
    meansig=mean([orisig0;dausig0]);meanmtx=ones(2,1)*meansig;
    orisig=orisig0./meanmtx;dausig=dausig0./meanmtx;
    dismtx=ppdist2(orisig,dausig);

    if (sum(diag(dismtx))<=sum(diag(fliplr(dismtx))))
        %link dau to ori
        x=(trk_rc(:,2)==tempdau(2));trk_rc(x,2)=tempori(2);
        x=(trk_rc(:,4)==tempdau(2));trk_rc(x,4)=tempori(2);
        trk_rc(tempori(2),[3,4])=trk_rc(tempdau(2),[3,4]);
        for f=tempdau(1):trk_rc(tempori(2),3)
            newsp{f}(tempori(2),:)=newsp{f} (tempdau(2),:);
        end
        mergelo(mergelo==tempdau(2))=tempori(2);
    else %link dau to mom, and link mom to ori, after split frame
        % link mom to ori
        x=(trk_rc(:,1)>=tempdau(1))&(trk_rc(:,2)==tempmom);trk_rc(x,2)=tempori(2);
        x=(trk_rc(:,3)>=tempdau(1))&(trk_rc(:,4)==tempmom);trk_rc(x,4)=tempori(2);
        trk_rc(tempori(2),[3,4])=trk_rc(tempmom,[3,4]);
        for f=tempdau(1):trk_rc(tempori(2),3)
            newsp{f}(tempori(2),:)=newsp{f} (tempmom,:);
        end
        % link dau to mom
    end
x=(trk_rc(:,2)==tempdau(2));trk_rc(x,2)=tempmom;
x=(trk_rc(:,4)==tempdau(2));trk_rc(x,4)=tempmom;
trk_rc(tempmom,[3,4])=trk_rc(tempdau(2),[3,4]);
for f=tempdau(1):size(trk_or,2);
    newsp{f}(tempmom,:)=newsp{f}(tempdau(2),:);
end
mergelo(mergelo==tempmom)=tempori(2);
mergelo(mergelo==tempdau(2))=tempmom;
end
orphanlo(tempdau(2))=1;

else % no split: add mom to ori
    trk_rc(tempori(2),3)=trk_rc(tempmom,3);
    if (trk_rc(tempmom,4)==trk_rc(tempmom,5))
        trk_rc(tempori(2),4)=trk_rc(tempori(2),5);
    else trk_rc(tempori(2),4)=trk_rc(tempmom,4);
    end
    for f=(tempori(1)+1):trk_rc(tempori(2),3)
        newsp{f}(tempori(2),:)=newsp{f}(tempmom,:);
    end
end
end
end

%% remove pseudo-merge
lo=(find((trk_rc(:,3)<size(trk_or,2))&(~orphanlo)&(trk_rc(:,4)~=trk_rc(:,5))));
clear trk_or
trk_rc(lo,4)=trk_rc(lo,5);

%% remove pseudo-split
lo=(find((trk_rc(:,1)>1)&(~orphanlo)&(trk_rc(:,2)~=trk_rc(:,5))));% find all split events
allmom=trk_rc(lo,[1,2,5]);momsz=size(allmom,1);% find all mothers
for cc=1:momsz
    siz_as=newsp{allmom(cc,1)}(allmom(cc,2),4);
siz_bs=newsp{allmom(cc,1)-1}(allmom(cc,2),4);
sig_as=newsp{allmom(cc,1)}(allmom(cc,2),3);
sig_bs=newsp{allmom(cc,1)-1}(allmom(cc,2),3);
if ((siz_as-siz_bs)/siz_bs>siz_vr(1))&&((sig_as-sig_bs)/sig_bs>sig_vr(1))
    % the split is not real; fix daughter's trk
    trk_rc(allmom(cc,3),2)=allmom(cc,3);
end

%%% remove orphans from newsp
for f=1:size(newsp,3)
    sz=size(newsp{f},1);
    newsp{f}=newsp{f}(~orphanlo(1:sz,:),:);
end

%%% relabel trk
oldlabel=(1:size(orphanlo,1))';
obsoletelabel=oldlabel(orphanlo==1);
oldlabel=oldlabel(orphanlo==0);
trk_rc=trk_rc(orphanlo==0,:);
% correct obsolete labels
for cc=1:size(obsoletelabel,1)
    obsofind=find(trk_rc(:,2)==obsoletelabel(cc));
    trk_rc(obsofind,2)=obsofind;
    obsofind=find(trk_rc(:,4)==obsoletelabel(cc));
    trk_rc(obsofind,4)=obsofind;
    obsofind=find(trk_rc(:,5)==obsoletelabel(cc));
    trk_rc(obsofind,5)=obsofind;
end
% relabel remains
for cc=1:size(oldlabel,1)
    trk_rc(trk_rc(:,2)==oldlabel(cc),2)=cc;
    trk_rc(trk_rc(:,4)==oldlabel(cc),4)=cc;
    trk_rc(trk_rc(:,5)==oldlabel(cc),5)=cc;
end

Filename: ppdist2.m

function mtx=ppdist2(mt1,mt2)
dm1 = size(mt1,2); dm2 = size(mt2,2);
sz1 = size(mt1,1); sz2 = size(mt2,1);
pt1 = ceil(sz1/1000); pt2 = ceil(sz2/1000);

if dm1 == dm2
  mtx = zeros(sz1,sz2);
  for row = 1:pt1
    for col = 1:pt2
      x1 = 1 + 1000*(col-1); x2 = min([1000*col,sz2]);
      y1 = 1 + 1000*(row-1); y2 = min([1000*row,sz1]);
      for dd = 1:dm1
        if dd == 1
          tmt = zeros(y2-y1+1,x2-x1+1);
        end
        tmt = tmt + (ones(y2-y1+1,1)*(mt2(x1:x2,dd)') - mt1(y1:y2,dd)*ones(1,x2-x1+1)).^2;
      end
      mtx(y1:y2,x1:x2) = sqrt(tmt);
    end
  end
end

Filename: rmduplicates.m

function [bestsp,best_rc] = rmduplicates(newsp, trk_rc, nucr)

% logic for duplicates
bestsp = newsp;
best_rc = trk_rc;
duplo = zeros(size(best_rc,1),1);
combi = unique(trk_rc(:,[1,3]),'rows'); scombi = size(combi,1);
roundnum = (scombi^2 + scombi)/2;
counter = 0; tempper0 = 0;

% main nested loop
for fl = 1:scombi
  ...
TRK1lo = find((best_rc(:,1)==combi(f1,1))&(best_rc(:,3)==combi(f1,2)));  
sz1 = size(TRK1lo,1); pt1 = ceil(sz1/1000);
for f2 = f1:scombi
    counter = counter + 1;
    temper = round(counter/roundnum*100);
    if (mod(temper,10)==0)&&(mod(temper0,10)~=0)
        disp(['Remove duplicates......',num2str(temper),'%'])
    end
    temper0 = temper;
end

%% find tracks starting f2 and ending f2
TRK2lo = find((best_rc(:,1)==combi(f2,1))&(best_rc(:,3)==combi(f2,2)));  
sz2 = size(TRK2lo,1); pt2 = ceil(sz2/1000);

%% find track pairs
for row = 1:pt1
    y1 = 1+1000*(row-1); y2 = min([1000*row,sz1]);
    for col = 1:pt2
        x1 = 1+1000*(col-1); x2 = min([1000*col,sz2]);
        % % get the distance matrix of each frame
        frnum = 0;
        for f = combi(f2,1):min(combi([f1,f2],2))
            % %
            if frnum == 0;
                DIS0 = ppdist2(bestsp{f}(TRK1lo(y1:y2),1:2),bestsp{f}(TRK2lo(x1:x2),1:2));
                [tempr0,tempc0] = find(DIS0<6*nucr);
                temper = temper0+y1-1; tempc = tempc0+x1-1;
                if (f1 == f2)
                    templo = (tempr<tempc);
                    temper = temper(templo); tempc = tempc(templo);
                end
                DIS = diag(DIS0(tempr-y1+1,tempc-x1+1));
                dis0 = (DIS==0);
                r = TRK1lo(tempr); c = TRK2lo(tempc);
                duplotemp = ((duplo(r)==0)&(duplo(c)==0));
                DIS = DIS(duplotemp); dis0 = dis0(duplotemp);
                r = r(duplotemp); c = c(duplotemp); % check only cells not removed yet
            end
        end
    end
end
for cc=1:size(r)
    disvec(cc)=pdist(bestsp{f}([r(cc),c(cc)],1:2));
end

dislo=(disvec<4*nucr);
disvec=disvec(dislo);DIS=DIS(dislo);dis0=dis0(dislo);
r=r(dislo);c=c(dislo);
DIS=DIS+disvec;
dis0=dis0+(disvec==0);
end
frnum=frnum+1;
end
% find track pairs
DIS=DIS/frnum;dis0=dis0/frnum;
DISLO=find(((DIS<1.5*nucr)&(dis0>0.5)));
r=r(DISLO);c=c(DISLO);

% correct track pairs
if ~isempty(r)
    % label duplicates
    duplo(c)=1;
    % relabel best_rc
    best_rc(r,3)=max([best_rc(r,3),best_rc(c,3)],[],2);
    for cc=1:size(r)
        best_rc(best_rc(:,2)==c(cc),2)=r(cc);
    end
    % average coordinates and values in bestsp
    for f=combi(f2,1):min(combi([f1,f2],2))
        mt1=bestsp{f}(r,:);mt2=bestsp{f}(c,:);
        bestsp{f}(r,:)=(mt1+mt2)/2;
    end
    % move track 2 to track 1 in bestsp
    if combi(f1,2)<combi(f2,2)
        for f=(combi(f1,2)+1):combi(f2,2)
            bestsp{f}(r,:)=bestsp{f}(c,:);
        end
    end
end
end
end
end

%%% remove duplicates and relabel best_rc
oldlabel=(1:size(duplo,1))';
obsoletelabel=oldlabel(duplo==1);
oldlabel=oldlabel(duplo==0);
best_rc(duplo==1,:)=[];

% correct obsolete labels
for cc=1:size(obsoletelabel,1)
    obsofind=find(best_rc(:,2)==obsoletelabel(cc));
    best_rc(obsofind,2)=obsofind;
    obsofind=find(best_rc(:,4)==obsoletelabel(cc));
    best_rc(obsofind,4)=obsofind;
    obsofind=find(best_rc(:,5)==obsoletelabel(cc));
    best_rc(obsofind,5)=obsofind;
end

% relabel remains
for cc=1:size(oldlabel,1)
    best_rc(best_rc(:,2)==oldlabel(cc),2)=cc;
    best_rc(best_rc(:,4)==oldlabel(cc),4)=cc;
    best_rc(best_rc(:,5)==oldlabel(cc),5)=cc;
end

%%% remove duplicates in bestsp
for f=1:size(bestsp,3)
    fsz=size(bestsp{f},1);
    bestsp{f}(duplo(1:fsz)==1,:)=[];
end
**Supplementary Code 2: Gradient measurement.**
This file contains the scripts for the measurement of Ca2+ and lipid sensor gradients. The users should copy the text from each script and save it as separate .m files with filenames specified as below, together with other scripts for data analysis. "main_script.m" should be used to generate gradient files of each cell, followed by "identify_leaders.m" to identify leader cells, and by "Get_round_summary" to generate quantification results.

**Filename: main_script**

```matlab
%% main script

clc;clear;close all
cwd='~/Users/FC/Dropbox_NTU/Dropbox/20130424_Huvec_P10_GCaMP6s/';
sf=fspecial('gaussian',5,1);sfd=fspecial('disk',2);
cd([cwd,'scripts/'])
tempwell='E08';
mkdir([cwd,'_Results'])

%% track cells
for sites=[0:5,12:17] % site
   % get file directory
   get_directories
   % loop to get values
   sss=cell(1,1,4);
   for f=1:4
      disp([sites,f])
      imaging_processing_get_numbers
      if f==3
         eyfpc0=eyfpc;ecfpc0=ecfpc;dapim0=dapim;dapic0=dapic;
      end
      sss{f}=double(sss{f});
   end
   % tracking
   sp=relocateD(sss);
   [newsp,trk_rc]=modifysp(sp);
   [bestsp,~]=rmduplicates(newsp,trk_rc,5);
```
identify_targets

TargetArea1= [tempwell,'.',num2str(sites)];
s=struct([]);
if ~isempty(bests{3})
    %% get corrected CFP and YFP images
    eyfpc=eyfpc0;ecfpc=ecfpc0;dapic=dapic0;
    [~,~,bgy]=ThreshImage(eyfpc);eyfpc=eyfpc-bgy;%eyfpc(eyfpc<0)=0;
    [~,~,bgc]=ThreshImage(ecfpc);ecfpc=ecfpc-bgc;%ecfpc(ecfpc<0)=0;
    [~,~,bgd]=ThreshImage(dapic);dapic=dapic-bgd;%dapic(dapic<0)=0;
    %correct bleedthrough from dapic to GCaMP6s
    dapic_g=mat2gray(sqrt(abs(dapic)));%sqrt
    th=graythresh(dapic_g);
    dapic_gm=im2bw(dapic_g,th);
    bleedr=eyfpc(dapic_gm)./dapic(dapic_gm);
    [~,th]=ThreshImage(dapic0);bleedr=ecfpc0(dapic0>th)./dapic0(dapic0>th);
    [~,~,bleedm]=ThreshImage(bleedr);
    eyfpc=eyfpc-bleedm*dapic;
    %get_cfp_yfp
    %% get masks
    msk_c=ThreshImage(sqrt(imfilter(abs(ecfpc0),sfd,'symmetric')));
    msk_y=ThreshImage(sqrt(imfilter(abs(eyfpc0),sfd,'symmetric')));
    m_or=msk_c&m_y;
    m_big=m_or;
    %m_big=bwselect(m_or,1*double(bests{3}(:,1)),1*double(bests{3}(:,2)));
    %m_big=imresize(m_sc,size(eyfpc));
    %dapim_sc=bwselect(dapim0,double(newsp{3}(:,1)),double(newsp{3}(:,2)));
    dapim_big=dapim0;dapic_big=dapic0;
    %dapim_big=imresize(dapim0,size(eyfpc));
    %dapic_big=imresize(dapic0,size(eyfpc));
    %correct background to 0
    %m_or2=msk_c&msk_y;
    %eyfpc=bgsubn(eyfpc,m_or2,16);
    %ecfpc=bgsubn(ecfpc,m_or2,16);
    %[~,~,bgy]=ThreshImage(eyfpc);eyfpc=eyfpc-bgy;eyfpc(eyfpc<0)=0;
    %[~,~,bge]=ThreshImage(ecfpc);ecfpc=ecfpc-bgc;ecfpc(ecfpc<0)=0;
eyfp = eyfp < 0; ecfp = ecfp < 0;

%% get round approximation
get_round
end

%%
save([cwd,'/_Results/',TargetArea1,'_s'],')
end

Filename: get_directories
dapidir1 = dir([cwd,tempwell,'_',num2str(sites),'_DAPI*.tif']);
ecfpdir1 = dir([cwd,tempwell,'_',num2str(sites),'_HeRed*.tif']);
eyfpdir1 = dir([cwd,tempwell,'_',num2str(sites),'_GFP*.tif']);

Filename: imaging_processing_get_numbers

%% read images
dapi1 = single(imread([cwd,dapidir1(f).name]));
dapif = imfilter(dapi1,fspecial('disk',2),'symmetric');
dapic = bgsub(dapif,16,0);
 [~,~,bg] = ThreshImage(dapic); dapic = dapic - bg;

eyfp1 = single(imread([cwd,eyfpdir1(f).name]));
eyfpf = imfilter(eyfp1,fspecial('disk',2),'symmetric');
eyfpc = bgsub(eyfpf,64,0);
 [~,~,bg] = ThreshImage(eyfpc); eyfpc = eyfpc - bg;

ecfp1 = single(imread([cwd,ecfpdir1(f).name]));
ecfpf = imfilter(ecfp1,fspecial('disk',2),'symmetric');
ecfpc = bgsub(ecfpf,64,0);
 [~,~,bg] = ThreshImage(ecfpc); ecfpc = ecfpc - bg;

%% alignment

%[cx,cy] = CalcJitter(imadjust(mat2gray(log(eyfpc+10000))),imadjust(mat2gray(log(ecfpc+10000))));
disp([cx,cy])

cx = -0.25;
cy = 0;
dapic=CropJitter(dapic,5,5,5,5,0,0);
eyfpc=CropJitter(eyfpc,5,5,5,5,0,0);
cefpc=CropJitter(ecfpc,5,5,5,5,cx,xy);

%% getmask
dapim=getdapimask(sqrt(dapic-min(dapic(:))),10);
dapil=bwlabel(dapim);
dapiring=imdilate(dapil,strel('disk',5))-dapil;

%% get xy coordinate and signal intensity
dapir=regionprops(dapil,'Centroid','Area','PixelIdxList');
dapir2=regionprops(dapiring,'PixelIdxList');
sz=size(dapir,1);
DD=zeros(sz,6);
for cc=1:sz
  sss{f}(cc,:)=[dapir(cc).Centroid,dapir(cc).Area,median(dapic(dapir(cc).PixelIdxList)),
               mean(eyfpc(dapir2(cc).PixelIdxList)),mean(ecfpc(dapir2(cc).PixelIdxList))];
end

%% get yfp and cfp threshold
tempseries=eyfpc(:);th_y=prctile(tempseries,75)+0.0*iqr(tempseries);
tempseries=ecfpc(:);th_c=prctile(tempseries,75)+1.5*iqr(tempseries);
sss{f}(:,7:8)=[sss{f}(:,5)>th_y,sss{f}(:,6)>th_c];

Filename: identify_targets.m
	sz=size(bestsp{1},1);szlo=bestsp{end}(1:sz,1)>0;
v=sqrt(sum((bestsp{end}(1:sz,1:2)-bestsp{1}(1:sz,1:2)).^2,2));
vlo=v>median(v);
ylo=(bestsp{1}(1:sz,7)+bestsp{end}(1:sz,7))==2;
clo=(bestsp{1}(1:sz,8)+bestsp{end}(1:sz,8))==2;
lo=(szlo&ylo&clo);
for f=1:4
  bestsp{f}=bestsp{f}(lo,:);
end

Filename: get_round.m

%% make round
RO=strel('disk',50,0);
MN=regionprops(getnhood(RO),'PixelIdxList');
MNS=MN.PixelList;
MND=MNS-51;
[MNT0,MNR]=cart2pol(MND(:,1),MND(:,2));
MNT=round(MNT0*180/pi);MNR=MNR/50;

%%% determine TS
TS0=double(1*bestsp{2}(:,1:2));
TS=double(1*bestsp{3}(:,1:2));TS_all=TS;
TS1=double(1*bestsp{4}(:,1:2));

%%% check speed and direction
d1=TS-TS0;d2=TS1-TS;d=d1+d2;
v=sqrt(sum((d).^2,2))/2;
ang1=atan2(d1(:,2),d1(:,1));ang2=atan2(d2(:,2),d2(:,1));ang=atan2(d(:,2),d(:,1));
angd=(acos(cos(ang2-ang1))*180/pi).*sign(asin(sin(ang2-ang1)));
lof=abs(angd)<90;
TS=TS(lof,:);
TS0=TS0(lof,:);
TS1=TS1(lof,:);
angm=ang(lof)*180/pi-90;
angd=angd(lof);
vm=v(lof);

%%% loop cells
k=1;% count of the cells stored
for i=1:size(TS,1)
    m_temp=bwselect(m_big,TS(i,1),TS(i,2));
    if sum(m_temp(:))>100
        m_box=regionprops(m_temp,'BoundingBox');m_box=round(m_box.BoundingBox);
        xo=[m_box(1)-32,m_box(1)+m_box(3)+32];
        yo=[m_box(2)-32,m_box(2)+m_box(4)+32];
        % exclude cells at the margin of the image
        if ((xo(1)>1)&&(xo(2)<size(ecfpc,2))&&(yo(1)>1)&&(yo(2)<size(ecfpc,1)))
            Nx=round(TS(i,1)-xo(1)+1);
            Ny=round(TS(i,2)-yo(1)+1);
        end
    end
end
ad3 = double(dapic_big(yo(1):yo(2),xo(1):xo(2)));  
ay3 = double(eyfpc(yo(1):yo(2),xo(1):xo(2)));  
ac3 = double(ecfpc(yo(1):yo(2),xo(1):xo(2)));  
%m = double(m_temp(yo(1):yo(2),xo(1):xo(2)));  
%  
% [~, th_ay3] = ThreshImage(ay3(ay3 > 0));  
% [~, th_ac3] = ThreshImage_ultrasen(ac3(ac3 > 0));  
%  
%m = (ac3 > th_ac3) & (ay3 > th_ay3);  
adm = double(dapim_big(yo(1):yo(2),xo(1):xo(2)));  

%% define cells  
figure(83);  
imshowc(sqrt(ay3.*(ay3>0)),sqrt(ac3.*(ac3>0)),ad3,1);axis on  
hold on;  
plot(TS_all(:,1)-xo(1)+1,TS_all(:,2)-yo(1)+1,'.k');  
plot(TS_all(:,1)-xo(1)+1,TS_all(:,2)-yo(1)+1,'sy');  
plot(Nx,Ny,'ok','markersize',12)  
[x,y] = getpts(83);  
close(83)  
if ~isempty(x)  
polymsk = roipoly(ay3,x,y);  
%m = double(m==1)&polymsk);  
m = double(polymsk);  
adm = double((adm==1)&polymsk);  

  
%%  
disp(i)  
r = imfilter(ay3./ac3,fspecial('disk',2),'symmetric');  
r = r.*m;r(~isfinite(r))=0;  
%
  
  
figure;  
subplot(1,2,1);imshowc(eyfpc,ecfpc,dapic_big,1)  
hold on;plot(TS(i,1),TS(i,2),'s','MarkerEdgeColor','k','MarkerFaceColor','w')  
   title(['num2str(i)/',num2str(size(TS,1))])  
xlabel(['angle: num2str(round(angm(i))),' degrees'])
r1=r;r1(isnan(r1))=0;
subplot(1,2,2);imshowc(ay3,ac3,r1.^0.25,1);
hold on;plot(Nx,Ny,'.','MarkerEdgeColor','k','MarkerFaceColor','w')
plot(round(TS0(i,1)-xo(1)+1),round(TS0(i,2)-yo(1)+1),'o','MarkerEdgeColor','k','MarkerFaceColor','w')
plot(round(TS1(i,1)-xo(1)+1),round(TS1(i,2)-yo(1)+1),'v','MarkerEdgeColor','k','MarkerFaceColor','w')
title(['speed: ',num2str(round(vm(i)*10)/10),' pixel/fr'])
xlabel(['rotate: ',num2str(round(angd(i))),' degrees'])
reply = input('Want this cell? [default:Yes; 2:No]: ');
close gcf
if (isempty(reply))||reply==2)
adm4=adm.*m;
ay4=ay3.*m;
ac4=ac3.*m;
r4=r.*m;

rotate the image
m5=imrotate(m,angm(i));
adm5=imrotate(adm4,angm(i));
ay5=imrotate(ay4,angm(i));
ac5=imrotate(ac4,angm(i));
r5=imrotate(r4,angm(i));

% check cell orientation and shape
cello0=regionprops(double(m5),'Orientation','Eccentricity','Area');
cello1=(cello0.Area>400)&&(median(ac5(m5))>cfilter);
%cello1=(cello0.Area>1500)&&(cello0.Eccentricity>0.5)&&(abs(tan((-cello0.Orientatio...<tan(45*pi/180));
%cello1=1;
if cello1==1

% convert to polar system
adm8=bwareaopen(bwperim(m5),50);
PC = regionprops(double(adm5), 'Centroid');
PN = regionprops(double(m5), 'PixelIdxList', 'PixelList');
Nx0 = PC.Centroid(1); Ny0 = PC.Centroid(2);
Z0 = log(r5(PN.PixelIdxList));
zlo = isfinite(Z0);
Z = Z0(zlo);
%Z = (Z0(zlo) - mean(Z0(zlo))) / std(Z0(zlo));
X0 = PN.PixelList(zlo, 1); X = X0 - Nx0;
Y0 = PN.PixelList(zlo, 2); Y = Y0 - Ny0;

PNb = regionprops(double(adm8), 'PixelList');
PNSb = PNb.PixelList;
xbb = PNSb(:, 1) - Nx0;
ybb = PNSb(:, 2) - Ny0;

[THETAb, RHOb] = cart2pol(xbb, ybb); THETA2b = THETAb * 180 / pi;
PNSbP = [THETA2b, RHOb]; PNSbP2 = sortrows(PNSbP);
PNSbP3 = [PNSbP2(end, :) ; PNSbP2(1, :)];
PNSbP3(1, 1) = PNSbP3(1, 1) - 360; PNSbP3(end, 1) = PNSbP3(end, 1) + 360;
PNSbP3(:, 1) = round(PNSbP3(:, 1));

% calculate the border radius
PNSbP4 = zeros(361, 2);
for j = -180:180
    lo = (PNSbP3(:, 1) == j);
    if sum(lo) > 0
        tl = max(PNSbP3(lo, 2));
    else
        ind = find(PNSbP3(:, 1) > j, 1, 'first');
        lo2 = (PNSbP3(:, 1) == PNSbP3(ind, 1)) ; d_r = max(PNSbP3(lo2, 2));
        lo3 = (PNSbP3(:, 1) == PNSbP3(ind - 1, 1)) ; d_l = max(PNSbP3(lo3, 2));
    end
    tl = d_l + (j - PNSbP3(ind, 1) - PNSbP3(ind - 1, 1)) ;
end
PNSbP4(j + 181, :) = [j, tl];
end
new_border = PNSbP4(MNT+181,2);  
new_radius = MNR.*new_border;  
[new_x, new_y] = pol2cart(MNT0, new_radius); 
new_x = round(new_x); new_y = round(new_y); 
RG = log(zeros(101,101));  
for j = 1:size(MNS,1)  
    tempx = new_x(j); tempy = new_y(j);  
    RG(MNS(j,2), MNS(j,1)) = mean(Z((X>=tempx-3)&(X<=tempx+3)&(Y>=tempy-3)&(Y<=tempy+3)));  
end  

%% figure1(RGB) 
figure; set(gcf,'color','w') 
subplot(2,2,1); hold on 
imshowc(ay4, ac4, 1) 

text(TS0(i,1)-xo(1), TS0(i,2)-yo(1), '1', 'Color', 'k') 
text(TS(i,1)-xo(1), TS(i,2)-yo(1), '2', 'Color', 'k') 
text(TS1(i,1)-xo(1), TS1(i,2)-yo(1), '3', 'Color', 'k') 

title(['speed: ', num2str(vm(i)), ' pixel/fr']) 
xlabel(['rotate: ', num2str(round(angd(i))), ' degrees'])

%% figure3(heat) 
subplot(2,2,3); hold on 
y1 = find(sum(m5,2)>0,1,'first'); y2 = find(sum(m5,2)>0,1,'last'); 
x1 = find(sum(m5,1)>0,1,'first'); x2 = find(sum(m5,1)>0,1,'last'); 
imagesc(r5(y1:y2,x1:x2), prctile(r5(m5==1), [1 99])); axis image ij 
Nx1 = Nx0-x1; Ny1 = Ny0-y1;  
plot(Nx1, Ny1, 'sw') 
plot([Nx1, Nx1], [Ny1-30, Ny1+30], ':w') 
text(Nx1-2, Ny1+10, '0', 'Color', 'w', 'FontSize', 14) 
text(Nx1-5, Ny1-10, '180', 'Color', 'w', 'FontSize', 14)
text(Nx1-12,Ny1,'-','Color','w','FontSize',18)
text(Nx1+6,Ny1,'+','Color','w','FontSize',18)
title(['ratio: ',num2str(median(r5(m5==1)))])
xlabel(['target: ',num2str(round(median(ay5(m5==1))))) 
' ref: 
',num2str(round(median(ac5(m5==1)))))
axis image

%% figure2 (whole)
subplot(2,2,2);hold on
imshowc(eyfpc,ecfpc,dapic_big,1)
plot(TS(i,1),TS(i,2),'sw')

%% figure4(round)
subplot(2,2,4);
imagesc(RG);colorbar;axis image

%% save data
print('-dpng',[cwd,'_Results/','TargetArea1','_',num2str(k),'.png'])
close gcf
s(k).v=vm(i);
s(k).rotate=angd(i);
s(k).YFP=median(ay5(m5==1));
s(k).CFP=median(ac5(m5==1));
s(k).r=median(r5(m5==1));
s(k).orien=atan(tan((cello0.Orientation-90)*pi/180))*180/pi;
s(k).eccen=cello0.Eccentricity;
s(k).Image=RG;
k=k+1;
end
% end
end
end
end

Filename: identify_leaders.m
clc
cwd='/Users/FC/Dropbox_NTU/Dropbox/20130424_Huvec_P10_GCaMP6s';
row_alpha={'C','D','E','F'};

for row=2:3
    for col=5:8
        for i=[0:5,12:17]
            load([cwd,'/_Results/',row_alpha{row},'0',num2str(col),'_',num2str(i),'_s']);
            for j=1:size(s,2)
                tempimg=imread([cwd,'/_Results/',row_alpha{row},'0',num2str(col),'_',num2str(i),'_',num2str(j),'.png']);
                figure(11);imshow(imresize(tempimg(59:329,677:1007,:),2))
                reply = input('Is this a leader cell? Y/N [N]: ', 's');
                if isempty(reply)
                    reply = 'N';
                end
                if reply=='Y' || reply=='y'
                    s(j).leader=1;
                else
                    s(j).leader=0;
                end
            end
            save([cwd,'/_Results/',row_alpha{row},'0',num2str(col),'_',num2str(i),'_s'],'s');
        end
    end
end

Filename: Get_round_summary.m
clc
%close all
ssum=[];k=0;
yyyfp=[];fretratio=[];
%cwd='\Users\FC\Dropbox_NTU\Dropbox\20130424_Huvec_P10_GCaMP6s';
cwd='H:\Axon_Database\20130424_Huvec_P10_GCaMP6s';
row_alpha={'C','D','E','F'};
target_ref='GCaMP6s/tdimer2';

%% summation
for row=2:3
    for col=5:8
        for i=[0:5,12:17]
            load([cwd,'/_Results/',row_alpha{row},'0',num2str(col),'_',num2str(i),'_s']);
            if ~isempty(s)
                for j=1:size(s,2)
                    disp([i,j])
                    if (s(j).r<0.27)&&(s(j).v>2.7)&&(s(j).YFP<7777)&&(s(j).YFP>=77)&&(s(j).CFP>=77)...
                        &&(abs(s(j).rotate)<=77)&&(s(j).leader==1)
                        
                        tempimg=imread([cwd,'/_Results/',row_alpha{row},'0',num2str(col),'_',num2str(i),'_',num2str(j),'.png']);
                        
                        figure(11);imshow(tempimg(59:329,677:1007,:))
                        reply = input(' Is this cell okay? Y/N [N]: ', 's');
                        if isempty(reply)
                            reply = 'N';
                        end
                        if reply=='Y' || reply=='y'
                            tt=s(j).Image;tt(isnan(tt))=0;
                            ti=tt;
                            % correct rotation
                            lo=double(isfinite(tt));
                            PN=regionprops(lo,Pixellist',PixelIdxList');
                            PNS=[PN.Pixellist,tt(PN.PixelIdxList)];
                            x00=PNS(:,1)-51;y00=PNS(:,2)-51;
                            [THETA,RHO]=cart2pol(x00,y00);
                            tempang=0;%(s(j).rotate/180*pi)/((s(j).v)^1);
% NEWTHETA = THETA + tempang;

[x11, y11] = pol2cart(NEWTHETA, RHO);

% for cc = 1 : size(x11, 1)

tx11 = round(x11(cc)) + 51; ty11 = round(y11(cc)) + 51;

txmin = max([tx11 - 1, 1]); txmax = min([tx11 + 1, 101]);

tymin = max([ty11 - 1, 1]); tymax = min([ty11 + 1, 101]);

tmatrix = tt(tymin:tymax, txmin:txmax);

% end

ti(y00(cc) + 51, x00(cc) + 51) = mean(tmatrix(isfinite(tmatrix)));

% k = k + 1;
ssum(:, :, k) = ti;

yyyfp(k) = s(j).YFP;
fretratio(k) = s(j).r;

% end
end
end
end
end
end

%% normalize

% ssum(isfinite(ssum)) = ssum(isfinite(ssum)) + 0.5; % for column 6
% ssum(isfinite(ssum)) = ssum(isfinite(ssum)) - 0.1; % for column 5
% ssum(isfinite(ssum)) = ssum(isfinite(ssum)) - 0.1; % for column 7

%% color map

msum = sum(ssum, 3) ./ sum(ssum ~= 0, 3);

% msum = median(ssum, 3);
msum = msum / log(2);
msum = msum - median(msum(isfinite(msum)));
figure;set(gcf,'color','w')
subplot(2,2,1);imagesc(msum,[-0.5 1]);axis ij
text([1,1],[6,95],{'Back','Front'},'color','y','fontweight','bold')
text([50.5,95,50.5,6],[95,50.5,6,95.5,90,'0\circ','90\circ','180\circ','90\circ'},'color','w','horizontalalignment','center')
xlabel(['n= ',num2str(k)]);ylabel('%')
title(target_ref)
colorbar

%% gradient (distance)

subplot(2,2,2);hold on
lo=double(isfinite(msum));
PN=regionprops(lo,'PixelList','PixelIdxList');
PNS=[PN.PixelList,msum(PN.PixelIdxList)];
tempx=PNS(:,2);tempy=PNS(:,3);bb=5;
plot([0,100],[0,0],':');
x=[];y=[];eyl=[];eyh=[];
for i=(1+bb/2):bb:(101 -bb/2)
    x=[x,i];
    lo=((tempx>=(i-bb/2))&(tempx<=(i+bb/2)));
    tempm=prctile(tempy(lo),[25,50,75]);
    y=[y,tempm(2)];
    eyl=[eyl,tempm(2)-tempm(1)];
    eyh=[eyh,tempm(3) -tempm(2)];
end
errorbar(x,y,eyl,eyh,'. -k')
xlabel('Back to Front (%)')
ylabel(['log(',target_ref,')'])

colorbar

%% gradient (angle)

subplot(2,2,3);hold on
x00=PNS(:,1)-51;y00=PNS(:,2)-51;
[THETA,RHO]=cart2pol(x00,y00);
THETA2=acos(cos(THETA -pi/2))*180/pi;
%THETA2=[THETA-2*pi;THETA;THETA+2*pi]*180/pi-90;
%RHO2=[RHO;RHO;RHO];
tempx0 = \text{THETA2}(\text{RHO} \leq 50 \& \text{RHO} \geq 40);
tempy0 = \text{PNS}(\cdot, 3);
tempy0 = \text{tempy0}(\text{RHO} \leq 50 \& \text{RHO} \geq 40); \%
\text{tempy} = \text{tempy0} - \text{prctile}(\text{tempy}, 20);
tempx = \text{unique}(\text{tempx0});
tempy = \text{tempx};
\text{for pp} = 1: \text{length}(\text{tempx})
    \text{tempy}(\text{pp}) = \text{mean}(\text{tempy0}(\text{abs}(\text{tempx0} - \text{tempx}(\text{pp})) < 10^{-10}));
\text{end}
\text{tempy} = \text{tempy} - \text{mean}(\text{tempy}(\text{tempx} > 150));

\text{bb} = 1;
\text{plot}(\text{tempx}, \text{tempy}, ',', ', \text{color}', [0.8, 0.8, 0.8]); \text{axis}([0 180 -0.34 0.17])
\text{plot}([0, 180], [0, 0]);
x = []; y = []; eyl = []; eyh = [];
\text{for i} = (\text{bb}/22): \text{bb}: (180 - \text{bb}/2)
    \text{x} = [x, i];
    \text{lo} = ((\text{tempx} \geq (i - \text{bb} * 20)) \& (\text{tempx} \leq (i + \text{bb} * 20)));
    \text{tempm} = \text{prctile}(\text{tempy(lo)}, [25, 50, 75]);
    \text{y} = [y, \text{tempm}(2)];
    \text{eyl} = [\text{eyl}, \text{tempm}(1)];
    \text{eyh} = [\text{eyh}, \text{tempm}(3)];
\text{end}
\text{plot}(\text{x}, \text{y}, 'r', 'linewidth', 3)
\text{plot}(\text{x}, \text{eyl}, 'r', 'linewidth', 1.5)
\text{plot}(\text{x}, \text{eyh}, 'r', 'linewidth', 1.5)
\text{xlabel}('\text{Front to Back (0\textdegree~180\textdegree})')
\text{ylabel}(['\text{log}_2(', target_ref, ')'])

\%
\text{m}_f = \text{zeros(size} (\text{ssum}, 3), 1); \text{m}_b = \text{zeros(size} (\text{ssum}, 3), 1);
\text{for cc} = 1: \text{size} (\text{ssum}, 3)
    \text{tempmtx} = \text{ssum}(91:101, :, cc);
    \text{m}_f(\text{cc}) = \text{mean} (\text{tempmtx}(\text{isfinite} (\text{tempmtx})));\n    \text{tempmtx} = \text{ssum}(1:11, :, cc);
    \text{m}_b(\text{cc}) = \text{mean} (\text{tempmtx}(\text{isfinite} (\text{tempmtx})));\n\text{end}
\text{[h, p]} = \text{ttest}(\text{m}_f - \text{m}_b);
text(10,0.1,['p = ',num2str(p)])

%% gradient (center to periphery)
subplot(2,2,4);hold on

tempx=RHO;tempy=PNS(:,3);bb=2.5;
plot(tempx,tempy,'.c');axis([0 50 -0.7 1.7])
plot([0,50],[0,0],':');
x=[];y=[];eyl=[];eyh=[];
for i=(0+bb/2):bb:(50-bb/2)
  x=[x,i];
  lo=((tempx>=(i-bb/2))&(tempx<=(i+bb/2)));
  tempm=prctile(tempy(lo),[25,50,75]);
  y=[y,tempm(2)];
  eyl=[eyl,tempm(2)-tempm(1)];
  eyh=[eyh,tempm(3)-tempm(2)];
end
errorbar(x,y,eyl,eyh,'. -k')
xlabel('Center to Border (pixel)')
ylabel(['log(',target_ref,')'])
Supplementary Code 3: Nuclear tracking.
This file contains the scripts for the measurement of cell migration parameters. The users should copy the text from each script and save it as separate .m files with filenames specified as below, together with other scripts for data analysis. "z_calcnuclei.m" is used to identify each nucleus in movies of cell migration. "z_tracenuclei.m" is used to generate the migration track of each cell. "z_calculate_healing.m" and "plot_healing" calculate the area of wound healing in sheet migration assays. "z_get_n_parameters_nocoor.m" is used to generate parameters from each nuclear track, including speed, persistence and directionality.

Filename: z_calcnuclei

```matlab
%% preparation
clc;close all;clear all;clear mex
time1=tic;
format short
jittersize=32;
r1=jittersize;r2=jittersize;
tempdir='H:\Axon_Database\20130406_Huvec_P4_Fura\Migration';
mkdir(fullfile(tempdir,'/data'))
SF=1;EF=49;%SF=starting frame, EF = ending frame
%sss=cell(8,12,EF); % used for storing cell coordinates
nucr=3;
wd=384;

%% loop
for i=3:6 %row indecies
    alp=char(64+i);
    for j=3:10 %column indecies
        %% setup tempwell
        if j<10
            tempwell=[alp,'0',num2str(j)];%alp= alphabetic letter, '0' , num2str fxn assigns a character to the value
        else tempwell=[alp,num2str(j)];
        end
        welldir=dir(fullfile(tempdir,'/tempwell/*DAPI*.tif'));
yfpdir=dir(fullfile(tempdir,'/tempwell/*YFP*.tif'));
wells=cell(1,1,EF-SF+1);
```
M=avifile([tempdir,'/data/',tempwell,'.avi'],'fps',12,'compression','none');

%% dapi
for f=SF:EF
    disp([i,j,f])%show row, column, frame
    time2=tic;

    %% reading images
    DAs_or=single(imread([tempdir,'/',welldir(f).name]));%DA = dapi, reads image
    if j>=18
        YFs_or=single(imread([tempdir,'/',yfpdir(f).name]));
    end

    %% correcting jitters
    DI1=imadjust(mat2gray(bgsub(log(imfilter(DAs_or,fspecial('gaussian'),'symmetric')),8*nucr,0)));%correct for background
    if f>SF
        [xx,yy]=CalcJitter(DI0,DI1);
        if (abs(xx)+abs(yy)>rx1)
            xx=0;yy=0;
            DI1=DI0;
        end
        x=x+xx;y=y+yy;
    else
        x=0;y=0;%store jitters data
        [~,th,bg]=ThreshImage(DI1(DI1>0 & DI1<1));
        th=prctile(DI1(DI1>0 & DI1<1),75)+1.5*iqr(DI1(DI1>0 & DI1<1));%find threshold for background
        msk=im2bw(DI1,th);
    end

    wndcenter0=regionprops(1-imdilate(msk,imshrink('disk',10*nucr)),'centroid');%find centroid of background
    wndcenter=round(wndcenter0.Centroid);
    wndcentery=median([wndcenter(2),1+jittersize+wd,size(DI1,1)-jittersize-wd]);
    upborder=wndcentery-wd;loborder=wndcentery+wd;
    ry1=upborder-1;ry2=size(DI1,1)-loborder;
end

DI0=DI1;
disp([x,y])
\%x0=round(x);y0=round(y);
\%ts=size(DAs_or);\%size of image
\%
\nx=0;y=0;

\%% cropping
\%DAs_or=DAs_or(1+ry1-y0:ts(1)-ry2-y0,1+rx1-x0:ts(2)-rx2-x0);
DAs_or=CropJitter(DAs_or,rx1,rx2,ry1,ry2,x,y);
if j>=18
    YFs_or=CropJitter(YFs_or,rx1,rx2,ry1,ry2,x,y);
end

\%\% image processssing
\%
\% DAPI
DAs_lo=mat2gray(sqrt(DAs_or));\%+mat2gray(sqrt(DAs_or));
DAs_bl=imfilter(DAs_lo,fspecial( 'disk',round(nucr/4)),'symmetric');
DAs_bs=bgsub(DAs_bl,3*nucr,0);
\%
\% YFP
if j>=18
    YFs_bl=imfilter(YFs_or,fspecial('disk',round(nucr/4)),'symmetric');
    YFs_bs=bgsub(YFs_bl,32*nucr,0);
end

\%% add frame
tempframe=imadjust(mat2gray(DAs_bs));
if 
  j>=18;tempframe(:,:,2)=imadjust(mat2gray(YFs_bs),stretchlim(mat2gray(YFs_bs),[0.05,0.995]));
  else tempframe(:,:,2)=tempframe;end
  tempframe(:,:,3)=imadjust(mat2gray(DAs_bs));
M=addframe(M,im2frame(tempframe));

\%% get data
DAs_ma=bwareaopen(getdapimask(DAs_bs,nucr),round((nucr+1).^2));
\%DAs_la=bwlabel(DAs_ma);

DAs_da=regionprops(DAs_ma,'Centroid','PixelList','PixelIdxList','Area','Perimeter','EquivDiameter');
XX=zeros(size(DAs_da,1),1);YY=zeros(size(DAs_da,1),1);

AC=zeros(size(DAs_da,1),1);%PP=zeros(size(DAs_da,1),1);DI=zeros(size(DAs_da,1),1);
DD=zeros(size(DAs_da,1),1); YF=DD;
for cc=1:size(DAs_da,1)
    XX(cc,1)=DAs_da(cc).Centroid(1);
    YY(cc,1)=DAs_da(cc).Centroid(2);
    AC(cc,1)=DAs_da(cc).Area;
    %         PP(cc,1)=DAs_da(cc).Perimeter;
    %         DI(cc,1)=DAs_da(cc).EquivDiameter;
    DD(cc,1)=mean(DAs_bs(DAs_da(cc).PixelIdxList));
    if j>=18
        YF(cc,1)=mean(YFs_bs(DAs_da(cc).PixelIdxList));
    end
end

%% filter data
if j>=18;wellsss{:,:,f-SF+1}=[XX,YY,DD,AC,YF];
else wellsss{:,:,f-SF+1}=[XX,YY,DD,AC];end
toc(time2)
end

save([tempdir,'/data/well_',num2str(i),'_',num2str(j),'.mat'],'wellsss')

%% end
toc(time1)


Filename: z_tracenuclei.m

%%
clear;close all;clc
time1=tic;
tempdir='H:\Axon_Database\20130406_Huvec_P4_Fura\Migration';
nucr=3;

%%
for i=3:6
    for j=3:10
%% read data

time2 = tic;

disp([i,j])

load([tempdir,'/data/well_',num2str(i),'_',num2str(j),'.mat'],'wellsss')

%% fix wellss

cellnum = zeros(size(wellsss,3),1);

for f=1:size(cellnum,1)
    cellnum(f)=size(wellsss{f},1);
end

upperlim = prctile(cellnum,75)+1.5*iqr(cellnum);
lowerlim = prctile(cellnum,25)-1.5*iqr(cellnum);

wellss1 = wellsss;

for f=1:size(cellnum,1)
    if (cellnum(f)>upperlim) || (cellnum(f)<lowerlim)
        if f>1
            wellsss1{f}=wellsss{f-1};
        else
            wellsss1{f}=wellsss{find((cellnum<upperlim) & (cellnum>lowerlim),1,'first')};
        end
    end
end

%% tracking

wellsp = relocateD(wellss1);

save([tempdir,'/data/well_',num2str(i),'_',num2str(j),'_wellsp.mat'],'wellsp','wellss1');
clear wellss1

[newsp, trk_rc] = modifyssp_beta(wellsp);

save([tempdir,'/data/well_',num2str(i),'_',num2str(j),'_newsp.mat'],'newsp','trk_rc');
clear wellsp

[bestsp, best_rc] = rmduplicates(newsp, trk_rc, nucr);

save([tempdir,'/data/well_',num2str(i),'_',num2str(j),'_bestsp.mat'],'bestsp','best_rc');
clear newsp trk_rc bestsp best_rc

toc(time2)
end
end

%%
toc(time1)
% clear; close all; clc
% tic; tempdir='H:\Axon_Database\20130406_Huvec_P4_Fura\Migration';
% gp=4; st=1; ed=49; trk=0;

%%
% n=zeros(8,12,ed-st); healing=zeros(8,12,ed-st);
for i=3:6
    for j=3:10
        for f=st:ed-1
            time2=tic;
            disp([i,j,f])
            load([tempdir,'\data\well_',num2str(i),'.',num2str(j),'.',bestsp,'.mat'],'bestsp')
            [healing(i,j,f-st+1),n(i,j,f-st+1)]=getmtxhealing(bestsp,f,f+1);
            toc(time2)
        end
    end
end
%
% save([tempdir,'\data\_H_data.mat'],'n','healing')
toc(time1)

%cwd='H:\Axon_Database\20130329_HUVEC_P9\Migration\data\';
% load([cwd,'\_H_data.mat'])
healfit=zeros(8,12);
% st=18; ed=48;

% for rows=3:6
%    for cols=3:10
a=healing(rows,cols,:)*960*2.5/2;a=a(:);
figure(23)
subplot(1,2,1);plot((st:ed)/4,a(st:ed),'. -')

%%
asum=zeros(size(a,1)+1,1);
for f=1:size(a,1)
asum(f+1)=sum(a(1:f));
end
subplot(1,2,2);plot((st:ed)/4,asum(st:ed),'. -')

%%
[b,stats]=robustfit((st:ed)/4,asum(st:ed));
%b=polyfit((st:ed)/4,asum(st:ed)',1);
healfit(rows,cols)=b(2);
end
end

%%
save([cwd,'_H_data.mat'],'n','healing','healfit')
%%
X = bestsp{st}(lo Fraser, 1); Y = bestsp{st}(lo Fraser, 2);
figure(42);
subplot(1, 2, 1); plot(X, Y, '.'); axis ij tight
title([rows, cols])
%
sep = kmeans(Y, 2, 'start', [1; 1024]);
ub = 2 * median(Y(sep == 1)) + 64; lb = 2 * median(Y(sep == 2)) - max(Y) - 64;
% th = mean([median(Y(sep == 1)), median(Y(sep == 2))]);
lo up0 = lo Fraser(Y < ub); [~, IX] = sort(Y(Y < ub), 'descend');
lo up = lo up0(IX(1:min([length(IX), round(nc/2)])));
lo dn0 = lo Fraser(Y > lb); [~, IX] = sort(Y(Y > lb), 'ascend');
lo dn = lo dn0(IX(1:min([length(IX), round(nc/2)])));
lo po = [lo up; lo dn];
subplot(1, 2, 1);
hold on; plot(bestsp{st}(lo po, 1), bestsp{st}(lo po, 2), 'or'); hold off
%
X0 = zeros(size(lo po, 1), (ed - st + 1)); Y0 = X0;
for f = st : ed
    X0(:, f - st + 1) = bestsp{f}(lo po, 1);
    Y0(:, f - st + 1) = [bestsp{f}(lo up, 2) - bestsp{f}(lo dn, 2)];
end
X = (X0 - X0(:, 1)*ones(1, ed - st + 1))*2.5;
Y = (Y0 - Y0(:, 1)*ones(1, ed - st + 1))*2.5;
%
figure(42);
subplot(2, 2, 2);
plot(median(X, 1), median(Y, 1));
hold on
plot(median(X(:, 1)), median(Y(:, 1)), '.r');
plot(median(X(:, end)), median(Y(:, end)), 'or')
hold off;
axis image;
title([n = ', num2str(size(X, 1))])
axis([-30,30,-10,300])

subplot(2,2,4);plot(X',Y');axis image
title('toward the wound')
xlabel(mean(abs(Y(:,end)))./mean(abs(X(:,end))))
%xlabel(std(atan2(X(:,end),Y(:,end)))*180/pi)
axis([-300,300,-150,600])

DIR_dev(rows,cols)=mean(abs(Y(:,end)))./mean(abs(X(:,end)));

%         coor=getmtxcoor(bestsp,st,ed,1,0);
%         disp(mean(coor))
%         COOR(rows,cols)=mean(coor);

%         [speed,lo]=getmtxspeed(bestsp,st,ed,1);
tempmtx=ppdist2(lo,lo_po);[x,~]=find(tempmtx==0);
disp(mean(speed(x))*2.5*4)
SPEED(rows,cols)=mean(speed(x))*2.5*4;

%         per=getmtxper(bestsp,st,ed);
disp(mean(per(x)))
PER(rows,cols)=mean(per(x));

%         ang=getmtxdir(bestsp,st,ed-st);
disp(mean(cos(ang(x))))
DIR_cos(rows,cols)=mean(cos(ang(x)));

%         save([cwd,'_N_data.mat'],'SPEED','PER','DIR_cos','DIR_dev','COOR')
end
end