Supplementary Information for
Leading edge elongation by follower cell interruption in advancing epithelial cell sheets

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Movies S1 to S12
Supplementary Materials and Methods

**Cell Culture.** Keratocyte sheets from the scales of African cichlids (*Maylandia lombardoi*) were cultured as previously described (1). Briefly, without sacrificing the fish, a few of their scales were removed and washed in culture medium (Leibovitz’s medium: L-15, L5520; Sigma-Aldrich, St Louis, MO) supplemented with 10% foetal calf serum (Nichirei, Tokyo, Japan) and antibiotic/antimycotic solution (09366-44: Nacalai Tesque, Kyoto, Japan). The scales were placed external side up on the floor of a square chamber (18 × 18 mm and 2 mm in depth), the bottom of which was made of a 24 × 24 mm coverslip (No. 1, Matsunami, Osaka, Japan) or on the surface of a substrate for traction force microscopy as described later. They were then covered with another small coverslip and allowed to adhere to the bottom coverslip for 30 min at 23 °C. Then, after removal of the upper coverslip, culture medium was added to the chamber and the scales were kept at 23 °C again for about 6 h to allow the cells to spread out from the scale. All the experiments were carried out in accordance with national guidelines and were approved by Yamaguchi University’s Animal Use Committee.

**Fixed Cell Staining.** Fixed cell staining was performed according to the methods described previously (2). Briefly, cells were fixed with 4% paraformaldehyde for 15 min, permeabilized with 0.1% Triton X-100 for 10 min, and blocked with 0.2% gelatine for 30 min. The cells were then incubated with primary antibody: mouse monoclonal vinculin (1:800 dilution, V9131, Sigma-Aldrich, St Louis, MO) and Alexa Fluor 488 phalloidin (0.33U/ml, A12379; Life Technologies, Carlsbad, CA) for 60 min. After several washes with 0.2% gelatine, the cells were incubated with secondary antibody: Alexa Fluor 546 Anti-mouse IgG (1:2,000 dilution, A11030, Life Technologies) for 60 min. When β-catenin or E-cadherin was simultaneously stained with vinculin, rabbit polyclonal β-catenin (1:100 dilution, GTX101435, GeneTex, Irvine, CA), rabbit polyclonal E-Cadherin (1:100 dilution, GTX100443, GeneTex), or rabbit polyclonal E-Cadherin (1:100 dilution, GTX125890, GeneTex), and Alexa Fluor 488 Anti-rabbit IgG (1:2,000 dilution, A-11034, Life Technologies), respectively, were used as the primary and secondary antibody. When myosin IIA was used for staining, the cells were permeabilized with 0.02% Triton X-100. Rabbit polyclonal myosin IIA (1:200 dilution, M8064, Sigma-Aldrich) and Alexa Fluor 546 Anti-rabbit IgG (1:2,000 dilution, A-11071, Life Technologies), respectively, were then used as the primary and secondary antibody.

**Traction Force Microscopy.** Traction force microscopy was performed according to the methods described previously (3), with minor modifications. A type of polydimethylsiloxane (CY52-276A and B; Dow Corning Toray, Tokyo, Japan) was used as the material for the elastic sheet. CY52-276A and B were mixed at a ratio of 7:10 by weight. A 60 - 70-mg aliquot of the mixture was spread on a 24 × 24 mm coverslip (No. 1, Matsunami, Osaka, Japan). After the mixture had solidified, the hardened substrates were kept in a hermetically sealed case with a 50-µl aliquot of liquid silane (3-aminopropyl triethoxysilane, Sigma-Aldrich) at 70 °C for 1 h, to attach the silane to the surface of the substrates by vapour deposition. A round chamber (16 mm in diameter × 2 mm in depth) was then assembled using the coated coverslip to form the bottom of the chamber. Orange fluorescent carboxylate-modified microspheres (200 nm in diameter; peak excitation and emission wavelengths of 540 and 560 nm, respectively; F-8809, Life Technologies) were attached to the surface of the substrates by employing the binding between the amino group in the silane and the carboxyl group in the microspheres. Finally, the surface of the sheet was coated with collagen (Cellmatrix I-C, Nitta Gelatin, Osaka, Japan).
The Young’s moduli of the elastic substrates were measured using the method of Lo and colleagues (4). Briefly, a steel ball (0.5 mm diameter, 7800 kg/m³) was placed on a substrate embedded with fluorescent beads. The Young’s modulus was calculated as \( Y = \frac{3(1-p^2)f}{4d^{3/2}r^{1/2}} \), where \( f \) is the force exerted on the sheet, \( d \) is the indentation, \( r \) is the radius of the steel ball, and \( p \) is the Poisson ratio (assumed to be 0.5 (5)). A typical value for the Young’s modulus was 5.3 kPa.

The image data were analysed using ImageJ software (http://imagej.nih.gov/ij/). The images of fluorescent beads were first aligned to correct any experimental drift using StackReg, an ImageJ plugin. The displacement field and the traction force field were then calculated using two plugins, one for particle image velocimetry and one for Fourier transform traction cytometry (6). The regularization parameter was set at \( 3 \times 10^{-10} \) for all traction force reconstructions.

**Blebbistatin treatment.** (±)-Blebbistatin (13186; Cayman, Ann Arbor, MI) was dissolved at 100 mM in DMSO and then diluted 4,000 times with culture medium. This blebbistatin medium was then added to the chamber, to the bottom of which the cell sheets had adhered, just after removal of the culture medium. After 30 min, the sheets in the chamber were used for experiments without removal of the blebbistatin medium.

**ImageJ Macro for Determination of the Number of Cell-Rows.**
```java
run("Set Measurements...", "area center redirect=None decimal=3");

x = newArray(2000);
y = newArray(2000);
INDEX = newArray(100);

R = 50;
Edge = 30;

for(k = 1;k <= nSlices;k++){  
    Array.fill(x, 0);
    Array.fill(y, 0);

    setSlice(k);
    run("8-bit");
    run("Analyze Particles...", "slice");
    run("RGB Color");

    nR = nResults();
    for(i = 0;i < nR;i++){
        x[i] = getResult('XM', i);
        y[i] = getResult('YM', i);
    }
}

run("Clear Results");
```
for(kk = 0; kk < 3; kk++){
    Array.fill(INDEX, 0);
    i = 0;
    index = searchEdge(nR, 1); // 0: Right, 1: Left
    if(index == 0){
        break;
    }
    INDEX[i] = index;
    X = x[INDEX[i]];
    Y = y[INDEX[i]];
}

for(i = 1; X < getWidth() - Edge; i++){
    index = searchNeighbor(X, Y, nR, 0); // 0: Right, 1: Left
    if(index == 0){
        break;
    }
    INDEX[i] = index;
    X = x[INDEX[i]];
    Y = y[INDEX[i]];
}

N = i;

for(; X > Edge;){
    index = searchNeighbor(X, Y, nR, 1); // 0: Right, 1: Left
    if(index == 0){
        break;
    }
    X = x[index];
    Y = y[index];
    flag1 = 0;
    for(i = 0; i < N; i++){
        if(INDEX[i] == index){
            flag1 = 1;
            break;
        }
    }
    if(flag1 == 0){
        INDEX[N] = index;
        N++;
    }
}

for(i = 0; i < N; i++){
    min = getWidth();
    for(j = 0; j < N - 1 - i; j++){
        if(x[INDEX[j] + 1] <= x[INDEX[j]]){
tmp = INDEX[j];
INDEX[j] = INDEX[j + 1];
INDEX[j + 1] = tmp;
}
}

i = 0;
X = x[INDEX[i]];
Y = y[INDEX[i]];
setResult("A", i, kk+1);
setResult("XL", i, X);
setResult("YL", i, Y);
setResult("DL", i, 0);
for(i = 1;i < N;i++){
    X = x[INDEX[i]];
    Y = y[INDEX[i]];
    xx = X - x[INDEX[i - 1]];
    yy = Y - y[INDEX[i - 1]];
    D = sqrt(xx * xx + yy * yy);
    setResult("A", i, kk+1);
    setResult("XL", i, X);
    setResult("YL", i, Y);
    setResult("DL", i, D);
}

saveAs("Results", "C:/Users/Desktop/LE/iwa"+k++"+kk+1+.csv");

if(kk == 0){
    setForegroundColor(255, 0, 0);
} else if(kk == 1){
    setForegroundColor(0, 255, 0);
} else if(kk == 2){
    setForegroundColor(0, 127, 255);
}

for(i = 0;i < N;i++){
    X = x[INDEX[i]];
    Y = y[INDEX[i]];
    floodFill(X, Y);
}

for(i = 0;i < N;i++){
    x[INDEX[i]] = 0.0;
    y[INDEX[i]] = 0.0;
showMessage("message");

saveAs("Jpeg", "C:/Users/Desktop/LEpic/+k+.jpg");
run("Clear Results");

}

}

showMessage("message");

}else{
    flag = 1;
}

}

close();

print("Macro Finished");

//end macro

function searchEdge(nR1, flag){
    index1 = 0;
    max1 = 0;
    for(i = 0; i < nR1; i++){
        if(max1 < y[i]){
            index1 = i;
            max1 = y[i];
        }
    }
    X1 = x[index1];
    Y1 = y[index1];

    if(flag == 0){
        for(;X1 < getWidth() - Edge;){
            index1 = 0;
            max1 = 0;
            for(i = 0; i < nR1; i++){
                xx = X1 - x[i];
                yy = Y1 - y[i];
                d = sqrt(xx * xx + yy * yy);
                if(d < R){
                    if(X1 < x[i]){ // condition for X1
                        if(max1 < y[i]){ // condition for y[i]
                            index1 = i;
                            max1 = y[i];
                        }
                    }
                }
            }
        }
    }
}

X1 = x[index1];
Y1 = y[index1];
X1 = x[index1];
Y1 = y[index1];

} else if(flag == 1){
    for(;X1 > Edge;){
        index1 = 0;
        max1 = 0;
        for(i = 0;i < nR1;i++){
            xx = X1 - x[i];
            yy = Y1 - y[i];
            d = sqrt(xx * xx + yy * yy);
            if(d < R){
                if(flag == 0){
                    if(X1 < x[i]){  
                        if(max1 < y[i]){  
                            index1 = i;
                            max1 = y[i];
                        }
                    }
                } else if(flag == 1){
                    if(X1 > x[i]){
                        if(max1 < y[i]){  
                            index1 = i;
                            max1 = y[i];
                        }
                    }
                }
            }
            X1 = x[index1];
            Y1 = y[index1];
        }
    }
}

return index1;

function searchNeighbor(X1, Y1, nR1, flag){
    index1 = 0;
    max1 = 0;
    for(i = 0;i < nR1;i++){
        xx = X1 - x[i];
        yy = Y1 - y[i];
        d = sqrt(xx * xx + yy * yy);
        if(d < R){
            if(flag == 0){
                if(X1 < x[i]){  
                    if(max1 < y[i]){  
                        index1 = i;
                        max1 = y[i];
                    }
                }
            } else if(flag == 1){
                if(X1 > x[i]){
                    if(max1 < y[i]){  
                        index1 = i;
                        max1 = y[i];
                    }
                }
            }
        }
    }
}

else if(flag == 1){
    if(X1 > x[i]){
        if(max1 < y[i]){  
            index1 = i;
            max1 = y[i];
        }
    }
} else if(flag == 1){
    if(X1 > x[i]){
        if(max1 < y[i]){  
            index1 = i;
            max1 = y[i];
        }
    }
} else if(flag == 1){
    if(X1 > x[i]){
        if(max1 < y[i]){  
            index1 = i;
            max1 = y[i];
        }
    }
}
if (max1 < y[i]) {
    index1 = i;
    max1 = y[i];
}

function hsv2rgb(h, s, v) {
    if (s == 0) {
        r = floor(v * 255);
        g = floor(v * 255);
        b = floor(v * 255);
    } else {
        phase = (h - floor(h)) * 6;
        ofs = phase - floor(phase);
        d1 = v * (1 - s);
        d2 = v * (1 - s * ofs);
        d3 = v * (1 - s * (1 - ofs));
        if (phase == 0) {
            r = floor(v * 255);
            g = floor(d3 * 255);
            b = floor(d1 * 255);
        } else if (phase == 1) {
            r = floor(d2 * 255);
            g = floor(v * 255);
            b = floor(d1 * 255);
        } else if (phase == 2) {
            r = floor(d1 * 255);
            g = floor(v * 255);
            b = floor(d3 * 255);
        } else if (phase == 3) {
            r = floor(d1 * 255);
            g = floor(d2 * 255);
            b = floor(v * 255);
        } else if (phase == 4) {
            r = floor(d3 * 255);
            g = floor(d1 * 255);
            b = floor(v * 255);
        } else if (phase == 5) {
            r = floor(v * 255);
            g = floor(d1 * 255);
            b = floor(d3 * 255);
        }
    }
    return [r, g, b];
}
b = floor(d2 * 255);
} else {
    r = floor(v * 255);
    g = floor(d1 * 255);
    b = floor(d2 * 255);
}

setForegroundColor(r, g, b);

References
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5. M. Dembo, T. Oliver, A. Ishihara, K. Jacobson, Imaging the traction stresses exerted by locomoting cells with the elastic substratum method. Biophys. J. 70, 2008–2022 (1996).
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Fig. S1. Localization of vinculin, β-catenin and E-cadherin. (A and B) Bottom (A) and middle (B, 0.6 µm above the bottom) optical sections of a fixed keratocyte sheet. Vinculin (green) and β-catenin (magenta). White arrows in B: vinculin (left) and β-catenin (middle) localization at the cell-
to-cell boundary on the actomyosin cables. White arrowheads in B: vinculin (left) and β-catenin (middle) localization at the boundary between leader cells and follower cells. (C and D) Bottom (C) and middle (D, 1 µm above the bottom) optical sections of a fixed keratocyte sheet. Vinculin (green) and E-cadherin (magenta). E-cadherin was stained using an antibody (GTX100443, GeneTex). White arrows in D: vinculin localization at the cell-to-cell boundary on the actomyosin cables. White arrowheads in D: vinculin localization at the boundary between leader cells and follower cells. (E and F) Bottom (E) and middle (F, 1.6 µm above the bottom) optical sections of a fixed keratocyte sheet. Vinculin (green) and E-cadherin (magenta). E-cadherin was stained using an antibody (GTX125890, GeneTex). White arrows in F: vinculin localization at the cell-to-cell boundary on the actomyosin cables. White arrowheads in F: vinculin localization at the boundary between leader cells and follower cells. Images in A - D, and E and F are typical of 3 and 6 sheets, respectively.
Fig. S2. Consecutive increase in cell number at the same or close location. (A and B) Trajectories of eight (a - h) leader cells in single sheets. Images are typical of 11 sheets. (C - E) The numbers of interval cells between the marked cells (a - h) in 3 h in Fig. 3A, and A and B in this figure. At 0 h, the number was 2 in all areas in all sheets (gray). The areas where the number of cells increased (arrows).
**Fig. S3.** Phase contrast sequential images of a single follower cell interruption to the leading edge. (A - C) Three examples other than Fig. 3E. In all cases, a stretched line (arrows) appears between the two leader cells (* at 0 min) and is broken between 42 and 47 min in A, 40 and 49 min in B and 21.5 and 26.5 min in C before the follower cell interruption is completed.
Fig. S4. Migration directionalities of individual cells in a keratocyte sheet. (A) Trajectories of all dotted cells in Fig. 4A. In each panel, the lengths of the trajectories are greater at the top than at the bottom. In other words, the migration velocities of the leader cells are fastest and the velocities of the follower cells, toward the rear of the sheet, are slower. At the same time, all the trajectories are almost straight. (B) Directionalities of all dotted cells in Fig. 4A. The directionalities of cells (a - e in Fig. 4B) are indicated by same-colour squares. The x-axis of B, representing the distance from the leading edge at 0 h, as a negative number. Directionality is expressed as the linear distance between the start and end points of migration over 2 h divided by the path length of the trajectory between the same points.
**Fig. S5.** Keratocyte sheets on the elastic substrate for traction force microscopy. (A and B) Bottom (A) and middle (B, 1.5 μm above the bottom) optical sections of a fixed keratocyte sheet. F-actin (green) and vinculin (magenta). *: Actomyosin cables. White arrowheads: vinculin localization at the cell-to-cell boundary on the actomyosin cables. Yellow arrowheads: vinculin co-localization with F-actin at the contour of follower cells. Images in A and B are typical of 4 sheets. (C and D) Velocities (C) and directionalities (D) of leader cells. Right in C and D: leader cells on the substrate for traction force microscopy; Left: on the coverslip. Error bars in C and D represent SEM. The p-values were calculated using Student’s t-test.
Fig. S6. Migration of keratocyte sheets treated with 25 µM blebbistatin. (A and B) Bottom (A) and middle (B, 0.8 µm above the bottom) optical sections of a fixed sheet. The sheet was fixed 30 minutes after the addition of blebbistatin. F-actin (green) and vinculin (magenta). There are no actomyosin cables at the boundary between the lamellipodium and the cell body of leader cells (areas surrounded by dotted lines), although F-actin and vinculin are colocalized at the cell-to-cell boundaries and appear white in the right-handed panels. Images in A and B are typical of 4 sheets. (C) Traction forces exerted by an advancing sheet. No large traction forces are shown under the lamellipodia of the leader cells. Images in C are typical of 3 sheets. (D) Sequential
images of a migrating sheet treated with 25 µM blebbistatin. The $a$ and $b$ are two arbitrarily selected leader cells. Leading edges between $a$ and $b$ are drawn with lines at each time in the right-hand panel. Time 0 h was defined as 30 minutes after the addition of blebbistatin. Images in $D$ are typical of 8 sheets. ($E$ and $F$) Velocities and directionalities of arbitrarily selected leader cells during 0 to 1.5 h. ($G$) The total numbers of leader cells between two arbitrarily selected cells ($a$ and $b$ in $D$) at 1.5 h. Two cells were selected so that the cell number between them was 17 at time 0 h (dotted line). Right in $E$ - $G$: treated with blebbistatin; Left: untreated. Error bars in $E$ - $G$ represent SEM. The $p$-values were calculated using Student’s $t$-test.
Fig. S7. Traction force distribution around two consecutively interrupting follower cells. The yellow lines in the phase images and the grey lines in the force images are on the boundary between the lamellipodium and the cell body where the actomyosin cables are present. At the beginning of the interruption (6 min), large traction forces (*) were detected at the boundaries between the first interrupting cell (arrows) and two adjacent leader cells on the actomyosin cables. The second cell (arrowheads) appears to accompany the first cell and interrupt into the leading edge (after 16 min). Large traction forces were detected under the right side of the first cell (**) and under the region from the left side of the first cell to the upper left of the second cell (***). Images are typical of 4 sheets.
**Fig. S8.** Actomyosin cable reconnection by an interrupting follower cell. (A - C) Three examples other than Fig. 5A. Top: F-actin visualized with HMRef. Bottom: DIC images (A) or phase contrast images (B and C). In all cases, a follower cell interposes itself between the two leader cells (*s). The actomyosin cables in A and B are broken between 5 and 8 min, and 8.5 and 19 min, respectively. The leader cells and the interrupting follower cell are then connected by the new actomyosin cables (arrowheads in A and B). In C, the original actomyosin cables (arrows in C) between the two leader cells move behind the new actomyosin cables (arrowhead in C) before they are broken (between 21 and 31.5 min).
Movie S1. Collective migration of keratocytes. The movie depicts the same keratocyte sheet as that shown in Fig. 1A and is shown 3,600 times faster than real time.

Movie S2. Three-dimensional schematic illustration of the leader and follower cells in a keratocyte sheet. The movie depicts the same illustration as that shown in Fig. 1K.

Movie S3. Collective migration of keratocytes with the trajectories of leader cells. Leader cells (a - h) in the first frame are marked every three cells. The movie depicts the same keratocyte sheet as that shown in Fig. 3A and is shown 3,600 times faster than real time.

Movie S4. Interruption of a single follower cell into the leading edge. The follower cell extends its lamellipodium between two leader cells (*s), and then a black line (arrow) that appears to be actomyosin cables break. The movie depicts the same keratocyte sheet as that shown in Fig. 3E and is shown 450 times faster than real time.

Movie S5. Interruption of a single follower cell into the leading edge. Three examples (A - C) other than Fig. 3E and Movie S4. The follower cell extends its lamellipodium between two leader cells (*s), and then a black line (arrow) that appears to be actomyosin cables break. The movies A, B and C depict the same keratocyte sheet as that shown in Fig. S3A, B and C, respectively. The movies are shown 450 times faster than real time.

Movie S6. Collective migration of keratocytes with the trajectories of the leader and follower cells. The left-hand frame shows the trajectories of 134 cells. Five typical cells are picked in the right-hand frame. The colours of the trajectories of each cell in the left and right movie are not the same. Anterior cells are faster. The movie depicts the same keratocyte sheet as that shown in Fig. 4 A and B and is shown 1,800 times faster than real time.

Movie S7. Traction forces exerted by a keratocyte sheet with no interrupting cells. The movie depicts the same keratocyte sheet as that shown in Fig. 4D and is shown 240 times faster than real time.

Movie S8. Collective migration of blebbistatin-treated keratocytes. The movie depicts the same keratocyte sheet as that shown in Fig. S6D and is shown 1,800 times faster than real time.

Movie S9. Traction forces exerted by a keratocyte sheet with an interrupting cell, shown by the arrow. The movie depicts the same keratocyte sheet as that shown in Fig. 4F and is shown 240 times faster than real time.

Movie S10. Live imaging of actomyosin cable reconnection by a single follower cell. Left: DIC images, right: F-actin. The cell interposes itself between two adjacent leader cells (*). The movie depicts the same keratocyte sheet as that shown in Fig. 5A and is shown 900 times faster than real time.

Movie S11. Live imaging of actomyosin cable reconnection by a single follower cell. Three examples (A - C) other than Fig. 5A and Movie S10. Left: DIC (A) or phase contrast images (B and C), right: F-actin. The cell interposes itself between two adjacent leader cells (*). Movies A, B and C depict the same keratocyte sheet as that shown in Fig. S8A, B and C, respectively. The movies are shown 450 times faster than real time.

Movie S12. Interruption of a single follower cell into the leading edge. The cell interposes itself between two adjacent leader cells (*). The movie depicts the same keratocyte sheet as that shown in Fig. 5E and is shown 150 times faster than real time. Immediately after the last frame of this movie, the sheet was fixed and F-actin and vinculin were stained as shown in Fig. 5 F and G.