

1 The complexity of keratocyte migration in salmon 2 explant cultures: initial results and future 3 prospects

4 **IDA S. OPSTAD,^{1,*} DEANNA L. WOLFSON,¹ BALPREET S.
5 AHLUWALIA,^{1,2} KRISHNA AGARWAL,¹ TORE SETERNES,² AND ROY A.
6 DALMO²**

7 ¹*Department of Physics and Technology, UiT The Arctic University of Norway, Tromsø, Norway*

8 ²*Department of Clinical Science, Intervention and Technology, Karolinska Institute, Stockholm, Sweden*

9 ³*Norwegian College of Fishery Science, UiT The Arctic University of Norway, Tromsø, Norway*

10 ^{*}*ida.s.opstad@uit.no*

11 **Abstract:** Intact skin is of uttermost importance for fish welfare. The fish skin provides an
12 environmental barrier and protects against invading pathogens. However, both pathogens and
13 physical insults cause skin wounds that are of major concern in modern fish farming. The
14 behavior and interactions between keratocyte cells and sheets of cells are not well understood.
15 The collective migration of keratocytes (skin epithelial cells) is of central importance for wound
16 healing in fish. In this study, we aimed to elucidate the complex wound healing process in fish
17 skin by studying in vitro cultures of these highly motile cells. Using explant cultures from
18 farmed Atlantic salmon (*Salmo salar*) and differential interference contrast microscopy (DIC),
19 we have captured the dynamics of sheets of cells from harvested fish scales and of individual
20 cells interacting in the cell sheet vicinity. In addition to direct contact, the cells were observed to
21 interact through long membrane tubes, turn, rotate, merge, and/or detach. Additionally, stationary
22 cells and cells moving on top of the cell sheets were observed. Cell sheets approaching one
23 another from different scales did not merge but dispersed.

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25 **1. Introduction**

26 The fish skin provides a barrier to environmental cues and against invading pathogens. Some
27 infectious pathogens and physical insults cause skin wounds [1], which have to be re-epithelialized
28 and remodeled quickly to sustain the barrier function. In fact, small wounds may be healed within
29 a few hours [2], depending on the environmental temperature. Keratocytes cover the dermis which
30 is composed of dense connective tissue, fibroblasts, pigment cells and blood capillaries [3] [4].
31 Intact skin is crucial for animals' defence against pathogens. In fishing industry, salmon louse
32 infestation is a huge concern for animal welfare. Non-medicinal treatments include physical
33 delousing processes such as freshwater bathing, warm water dips, use of lasers to kill individual
34 lice on the fish, mechanical removal of parasites by soft brushes and/or high-pressure pumps [5].
35 Such physical treatments are likely to affect the fish skin integrity as well as the activity of
36 keratocytes, which are important for tissue remodeling. The migratory behavior of keratocytes
37 (skin epithelial cells) is a key feature during wound repair. It has been shown that the speed of
38 the fish moga (*Hypsophrys nicaraguensis*) keratocytes is approximately 15 µm/min (23 °C) in
39 vitro [6]. This finding is similar to the previous results presented by [7] where the velocity of
40 black tetra (*Gymnocorymbus ternetzi*) keratocytes was calculated to be 15 µm/min. There are
41 numerous studies on fish keratocytes that have described the migratory features of individual
42 cells [8] [9] [10]. However, the far more complex behavior of sheets of cells – as found in fish skin
43 – is comparatively unexplored. Here, cells are seen to transition between *leader* and *follower* cell
44 morphology. The leader cells at cell-sheet edges typically exhibit the characteristic keratocyte

45 fan-shape and appear to determine the direction of the entire sheet of cells. The follower cells are
46 within the cell sheet and follow the movement of the neighboring cells [11] [12].

47 Using explant cultures from farmed Atlantic salmon (*Salmo salar*) and differential interference
48 contrast microscopy (DIC), we have observed both the advance of sheets of cells coming off the
49 harvested fish scales and the interactions of individual cells detaching and recombining both with
50 other cells and with the cell sheet. In this work, we show videos of Atlantic salmon keratocytes
51 acquired at room temperature, and discuss their complex dynamics as observed in scale explant
52 cultures.

53 **2. Materials and Methods**

54 *2.1. Cell harvesting and cultivation*

55 The imaged cells were primary epidermal skin cells (mainly keratocytes) obtained from Atlantic
56 salmon scales. The scale harvest was conducted with disinfected tweezers on farmed salmon
57 in air, killed with a blow to the head. This method is allowed according to the Norwegian
58 Regulations for use of animals in experimentation ([https://lovdata.no/dokument/SF/
59 forskrift/2015-06-18-761#KAPITTEL_10](https://lovdata.no/dokument/SF/forskrift/2015-06-18-761#KAPITTEL_10)) and complies with the corresponding
60 EU legislation - Directive 2010/63/EU ([http://data.europa.eu/eli/dir/2010/
61 63/obj](http://data.europa.eu/eli/dir/2010/63/obj)). The scales were placed on #1.5 glass-bottom dishes (734-2904, VWR) and dried
62 on for about 1-2 minutes (to make the scales stick to the dish) before adding antibiotic buffer
63 solution: Hank's Balanced Salt Solution (HBSS, Corning, #21-023-CM) with 100 IU/mL
64 Penicillin-Streptomycin (P/S, Sigma, P0781) and 1 µg/mL Amphotericin B (PanReac AppliChem,
65 #A7009). The short dry period did not appear to damage the harvested cells. The cells were
66 kept at 4°C in a watertight box, replacing the old medium with fresh every 2-3 days. Scales
67 that loosened during cell culture and sample preparation were removed from the culture dish.
68 Before imaging, loose cells and mucus were washed away by changing the media to a fresh HBSS
69 antibiotic solution.

70 *2.2. Image acquisition*

71 Imaging was performed at room temperature using a DeltaVision Elite microscope (GE Healthcare)
72 equipped with a DIC module, and an Olympus 10X, 0.4NA or 20X, 0.75NA objective. Unless
73 otherwise stated, the DIC module with the 10X objective was used. The microscope focus
74 together with the contrast of the DIC module were manually optimized to maximize the visibility
75 of the lamellipodia of leading-edge keratocytes adherent to the glass-substrate. For long-duration
76 time-lapses, the keratocytes were maintained in sharp contrast with help of the microscope's
77 inbuilt focus maintenance system. The exact acquisition parameters for all raw image data are
78 available with the published dataset [13].

79 *2.3. Image processing and analysis*

80 All image processing (linear contrast adjustment, file conversion, insertion of scale bars, etc.)
81 was conducted in Fiji [14]. The stitched images (combining multiple smaller field-of-views, files
82 labeled STC) were generated using the Fiji plugin Grid/Collection Stitching [15].

83 **3. Results**

84 With the explant culture preparation described under Material and Methods, two different image
85 acquisition schemes were conducted over 0-4 days after sample harvesting:

86 I. 2 min time-lapses (for up to about 24 hours) often consisting of multiple adjacent images
87 together covering a large sample area (e.g. 3 × 3 mm²) where cells (or sheets of cells)
88 were starting to come off the scale (i.e., a beginning cell avalanche).

89 II. 5 s time-lapses (for up to 14 hours) covering a single field-of-view (either $668 \times 668 \mu\text{m}^2$
90 for 10X lens, or $334 \times 334 \mu\text{m}^2$ using a 20X lens) close to a scale edge.

91 Approach I enabled an overview of the scale avalanche (large sheet of keratocytes) dynamics
92 over a relatively long time-frame and large field-of-view to be captured, while at the same time
93 achieving sufficient image resolution and contrast to capture individual cell dynamics, including
94 the very thin lamellipodia of the cell sheet leader cells, as demonstrated in Figure 1. Video 1
95 shows an overview of the full avalanche dynamics. Similarly, Video 2 shows an overview of a
96 different sample, but here of two approaching avalanches from two different salmon scales (but
97 from the same fish). Surprisingly, such avalanches were not found to merge but rather tended
98 to disperse or avoid each other. Considering that cell avalanches *in vivo* are supposed to cover
99 skin wounds, we expected to see the avalanches merging. The temperature or phototoxicity for
100 imaging could be important contributors to this behavior, especially since we found multi-scale
101 dishes (kept at 4°C protected from light) to eventually be fully covered by cells after enough days
102 on a dish.

103 With approach II, we were better able to study cell-cell interaction and details of their complex
104 behaviour. The following section outlines some interesting observations regarding individual
105 and inter-cell interactions following approach II.

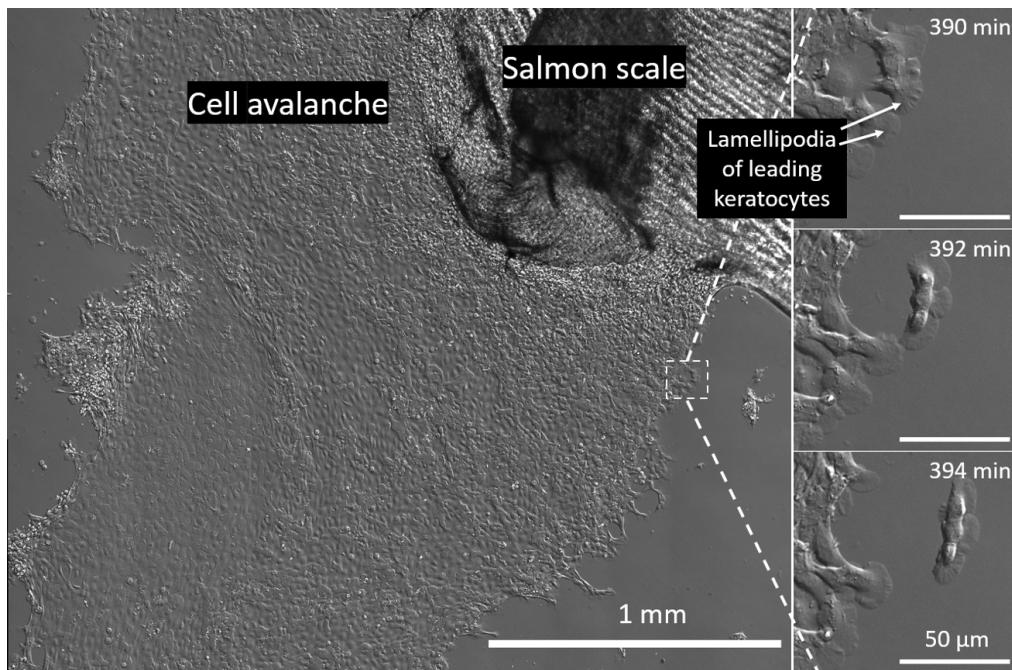


Fig. 1. Live imaging of explant scale culture of Atlantic salmon 3 days after harvest. Epithelial cell avalanches (as seen coming off the scale in the main panel) are important in the wound healing mechanisms of fish skin. This approach enabled an overview of the explant culture cell avalanche to be captured at the same time as achieving sufficient resolution and contrast to investigate individual cell and lamellipodium dynamics (right panels). The left panel is a tiled view of 5×6 DIC images. The cell avalanche shown had already been imaged for 390 min (every 2 min) at room temperature. No nutrition was provided to the culture medium (antibiotic-supplemented HBSS).

106 **3.1. Cell dynamics (5 s time-lapse)**

107 Different from the unidirectional motion normally described following keratocyte analysis,
108 our explant culture data show a broad range of different cell behavior and motion patterns, as
109 outlined in Figure 2. Some changes in propagation direction are clearly induced by direct contact
110 with other cells or membrane tubes (see Video 3), but also single cells without no apparent
111 direct cell contact were found to change the direction of propagation, as can be seen from the
112 time-lapse displayed in Figure 3 and Video 4, where a cell turns following apparent folding of its
113 lamellipodium. Figure 4 and Video 5 display a rotating cell (or collection of cells), seemingly
114 caused by a combination of keratocyte cells migrating in opposite directions.

115 Interestingly, also cells moving independently on top of the keratocyte cell sheet were observed
116 (Video 6 and indicated by yellow arrows in Figure 5). These cells appear different from keratocyte
117 cells in migratory behavior and morphology, although they have not been further characterized
118 beyond the visual observation.

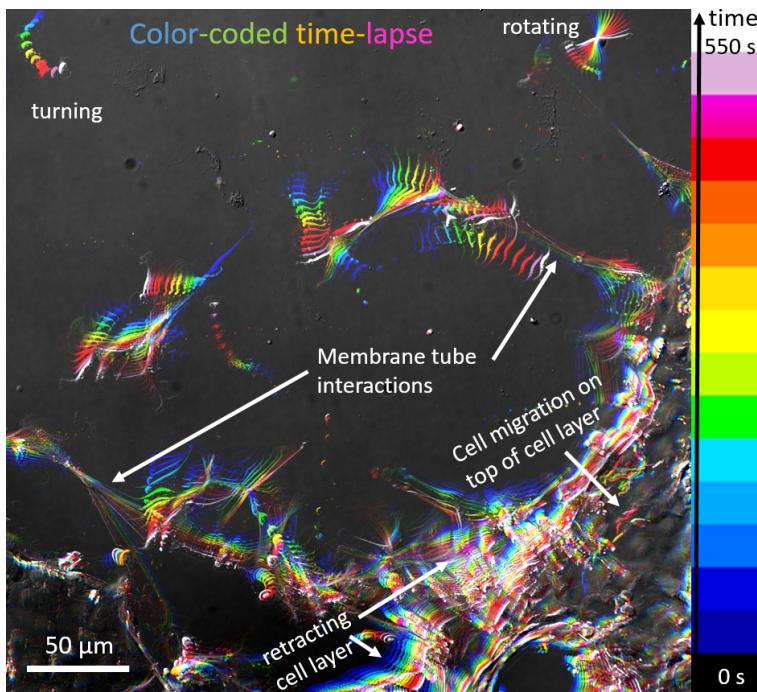


Fig. 2. Color-coded time-lapse of keratocyte video: each color represents a different time-point according to the sequence indicated by the color bar (50 s between each color). Cells undergo several different dynamical patterns: cells are turning, rotating, interacting through membrane tubes, and cells are moving on top of the retracting cell layer. Scale bar: 50 μ m. The figure was created with help of a Temporal-Color Coder script, provided by Kota Miura at the Centre for Molecular and Cellular Imaging, EMBL Heidelberg, Germany.

119 **4. Discussion**

120 We have in this work investigated the ability of label-free DIC microscopy to study the dynamics
121 of Atlantic salmon keratocyte cells using explant cultures. The collective behavior of keratocyte
122 cells is highly relevant for further understanding of the defense mechanisms of fish skin. Rather
123 than providing conclusive measurements regarding individual cell speed, morphology or chemical

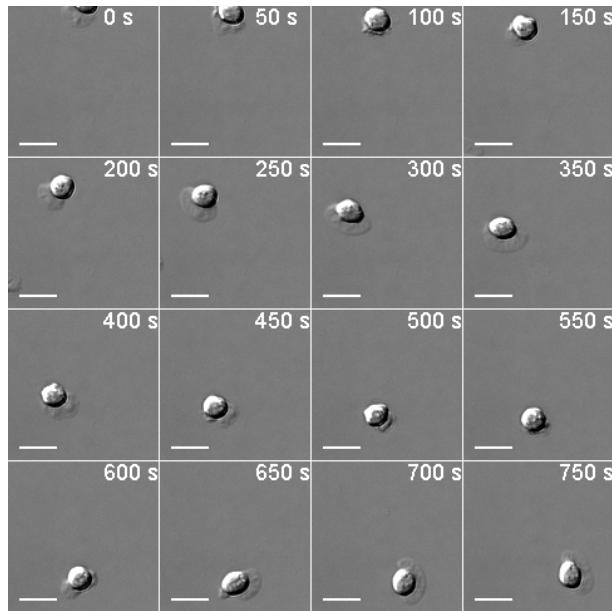


Fig. 3. Time sequence of a cell balling up and turning. Scale bars: 20 μ m.

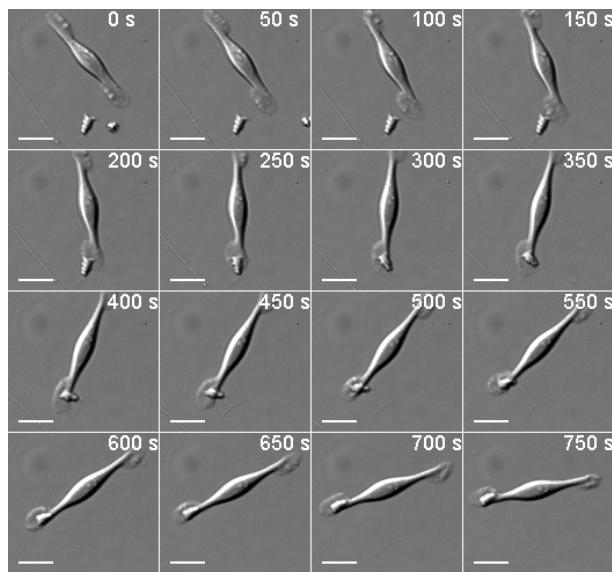


Fig. 4. Time sequence of a rotating cell collecting and transporting along a large particle from 200 s – 750 s. Scale bars: 20 μ m.

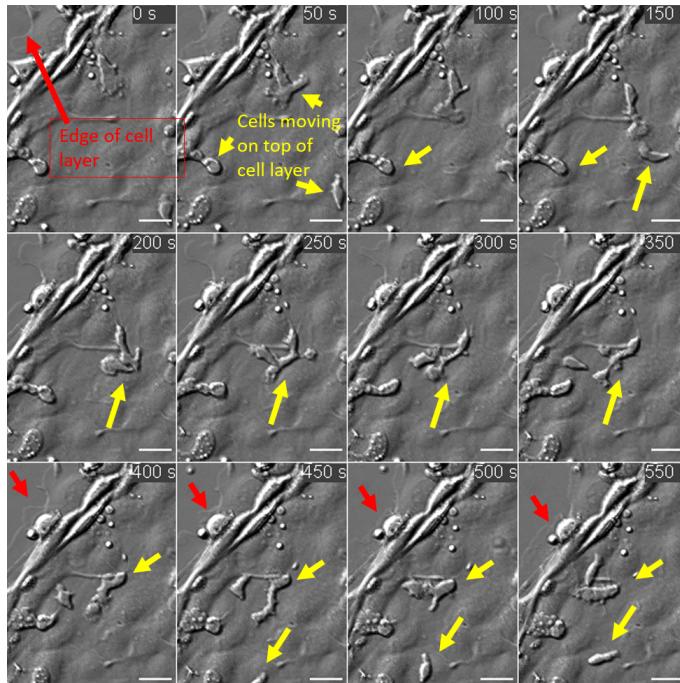


Fig. 5. Time sequence of cells moving on top of a connected but dynamical cell layer. The red arrows point at the edge of the cell sheet, while the yellow arrows are indicating cells on top of the cell layer moving in an independent fashion compared to the cells sheet beneath. Scale bars: 20 μ m.

124 constitution, this report aims to highlight the suitability of DIC and label-free microscopy methods
125 for the study both of primary cells and collective cell dynamics.

126 The collective behavior of cells over different length scales and image resolutions is a
127 challenging target for both manual and automated analysis. The cells are constantly moving,
128 interacting and changing their shape and propagation direction. The cell image contrast is poor
129 compared to the surrounding medium. Although the image contrast can in general be improved
130 using fluorescence imaging techniques, the requirement of fluorescent labeling and the associated
131 increase of phototoxicity alters the biological system and significantly reduces the possible
132 duration of time-lapse studies and the ability to follow a cell population over time.

133 From our videos, many interesting observations can be made. The large field-of-view time-
134 lapse studies (imaging many adjacent panels every 2 min over several hours), indicate that
135 approaching avalanches in general do not merge, but rather dispersed or avoid each other, even
136 when all cells originated from the same fish. This was observed three out of three times but could
137 be related to the temperature or light exposure during imaging. Observing meeting avalanches
138 was a relatively rare event as it is hard to predict exactly where from a scale an avalanche will
139 emanate and two different scales must be placed at the right distance from one another for
140 observation. We also noted that the progression was very slow – or even completely halted – of
141 the scale explant cultures while kept at 4°C compared to room temperature. This slow keratocyte
142 migration could be a key contributor to the increased presence of skin wounds in farmed fish
143 during the winter season [16].

144 The single field-of-view videos (imaged every 5 s) were particularly suitable for revealing
145 single-cell dynamics and their interactions. The DIC microscope provided relatively good
146 contrast of even the very thin lamellipodium and long membrane tubes. Cells were observed to

147 turn, rotate, and to move on top of the cell sheets. The microscope's in-built focus lock system
148 was important to maintain the cells and their thin lamellipodium (extending on the glass substrate)
149 in focus during several hours of imaging.

150 The videos trigger many questions concerning keratocyte cells and fish biology. Which factors
151 determine the keratocyte path of propagation? Do the cells divide in culture or are they just
152 advancing down from the harvested scales? From where do they derive their energy? Most of
153 our data were acquired of cells in a saline solution only (HBSS with antibiotics), without any
154 nutritional supplements commonly used in cell growth medium and fetal calf serum. Without
155 any growth medium supplements, the cells were observed active for up to 9 days after harvest,
156 contained in only HBSS medium and antibiotics. The cells were kept at 4°C until imaging, then
157 at room temperature. Future studies will investigate for how long after harvest keratocyte cells
158 remain active and further how conditions like temperature or cell culture medium supplements
159 affect the durability of salmon skin cells.

160 The analytical complexity of label-free bioimage analysis combined with the enormous
161 variability of motion pattern of salmon epithelial cells make it essential to develop suitable tools
162 for automated analysis. This will enable a rigorous quantitative assessment of the cell population
163 effects of e.g. different chemicals, pathogens, nutrients or temperature changes.

164 In summary, we have shown label-free DIC microscopy as a promising tool to study both
165 single cells and collective dynamics of salmon scale explant cultures. We hope our videos will
166 inspire many future studies of the collective behavior of fish skin cells and the development of
167 analysis software that can accelerate the quantitative assessment of such and similar label-free
168 bioimage videos. The data generated in this study is available on the research data archive
169 DataverseNO [13], and includes both the full image data as well as easy to visualize down-sampled
170 movies to enable a quick overview of the extensive time-lapse data.

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174 **Disclosures.** The authors declare no conflicts of interest.

175 **Data availability.** Data underlying the results presented in this paper are available from DataverseNO [13].

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