Interplay between chemical and mechanical guidance during collective cell migration

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Background

Since the discovery of granulocyte's ability to sense and respond to concentration gradients of specific signaling molecules (1), it was clear that the establishment of polarity in cell migration was a fundamental event. Experimentally a chemoattractant-loaded micro-needle can be placed in proximity of a single immune cell (1): the diffusion of the chemical signal immediately causes the reorganization of the cell migratory machinery enabling the cell to actively migrate in the direction of the chemical source. Directed migration is not an exclusive behavior of immune system cells. Mesenchymal-like cells often show great migratory ability as single units (2), participating in a numerous of physiological (tissue homeostasis) (3) as well as pathological (tumor metastasization) (4) processes. The differentiation of a cell into a migratory unit is usually explained in a comprehensive conceptual framework named Epithelial-to-Mesenchymal Transition (EMT), a theory that explains the modifications of the cell-substrate and the cell-cell adhesion molecules that take place in a cell in order for it to become migratory (5). Recent findings have shown that the understanding of the role of migration needs to be expanded, as the phenomenon of mesenchymal migration represents only a small subset of pathophysiological cases. In fact, cells often move together in cohesive clusters during events observed both in development and postnatal life. In physiological conditions, collective cell migration has been proved to be central for proper morphogenesis (6), wound healing, and tissue regeneration (7). Moreover, collective cell migration is the active mechanism observed in the early phases of tumor mass invasion (8).

Similarly to single cell chemotaxis, it has been shown that cell cluster movements can also be polarized by secreted chemoattractants (9). The directionality of migration is likely to be obtained through an integration of external chemoattractant and chemorepellent signals, both soluble and extracellular matrix bound factors expressed by surrounding cells (10-11). The presence of chemorepellent signals produced by specialized cells is supposed to generate predetermined pathways for migration, while the release of a chemoattractant signal generates a concentration gradient across the tissues. Ultimately the responsive cell cluster is able to sense and interpret the

chemical signals of the microenvironment, therefore responding by actively migrate along the chemotactic gradient.

However, recent works have shown that this picture is incomplete. Indeed, it has been proved that the polarity of collective migration is not only imposed by defined chemoattractant-receptor axis, but is also strongly regulated by cell-cell junction within the cell cluster (12). This finding is well surprising, as it bring to the attention the fact that also mechanical connections between cells are important to integrate a chemotactic clue, but unfortunately a detailed mechanistic model remains undefined. Cluster cohesiveness is the result of cell-cell interactions mediated by tight/adherent junction proteins and complexes. Some of these proteins (e.g. Cadherins) have been found not only to put cells in physical connection, but also to transmit forces and transduce them into biochemical signals (mechanotransduction) (13). This process is likely to involve a number of proteins recruited at the cytoplasmatic domain of cadherins (alpha and beta catenin, together with p120 and vinculin are supposed to be central for this role [13]). The force applied at the adherent junction is sufficient to cause conformational changes in one or more of the members the complex, event that can unfold certain cryptic binding sites leading to signal transmission across the cell.

A very clear example of this emerging paradigm is proposed to be *Xenopus leavi* Neural Crest Cell (NCC) migration (12), a mesenchymal tissue induced during embryo gastrulation and responsible to originate part of the adult peripheral nervous system. During Xenopus embryo morphogenesis NCCs begin their migration as a cohesive cluster of cells along a pathway defined by surrounding chemorepellent ephrins and semaphorines molecules. Apparently the directionality of this migratory event is driven by secreted chemoattractants, among which Stromal Derived Factor 1 (SDF1) has being confirmed to act as main source of migration activator, triggering response through its physiological receptor CXCR4. NCCs are attracted by SDF1 and promptly move together as a cluster in the direction of SDF1 source, but the mechanism of directed migration is readily disrupted upon down-regulation of the cell-cell junction molecule N-cadherin. To interpret these findings, it was proposed that SDF1 triggers the formation and stabilization of migratory protrusions by activating actin polymerization factor RAC1, while N-cadherin orchestrates the disposition of these protrusions across the cell cluster by activating RAC1 inhibiting factor RHO (14). In this way only cells with a free edge can develop a sufficiently stable lamellipodia (Contact Inhibition of Locomotion), and the cells at the edge closer to the source of SDF1 will make stronger, more active protrusions, giving rise to biased, directed cell migration.

Hypothesis and aim

This proposal is based on the hypothesis that force transmission across cell-cell junctions is required for efficient sensing and responding to a chemical gradient. To test this hypothesis our aim is to develop a novel experimental model to measure intercellular forces during chemotaxis of NCCs explants.

The specific aims addressed so far are the following:

1. To study Traction Forces migration of Neural Crest Explants in the absence of a chemotactic gradient.

    1.1 To optimize the mechanical condition (e.g. gel stiffness) ideal for the measurement of traction forces generated by NC cells during migration.

    1.2 To optimize a reliable method of generation of a chemotactic gradient for NC explants.

2. To study the role of candidate mechano-transducing molecules during chemotactic response of NC explants.

    2.1 To test the effects of silencing/perturbing molecules on NC explants survival and migration on PAA-gel.

    2.2 To develop new algorithms for the quantification and analysis of time-lapse results.

Methodology

Ex-vivo culture of NCCs explants

Early stage *X.leavi* embryos (2-4 cell stage) were injected with 10ng Fluorescein-Dextran (FDX) for staining/computational purpouse. NCCs clusters of about 100 um of diameter are obtained from stage 16-19 embryos by dissection and mechanical disgregation, and cultured in DFA.

Polyacrylamide (PA) gels for traction microscopy
Glass-bottom dishes were silanized with Bind-Silane solution (1:1:14 EtOH: Silane-plus:acetic acid) for 30 minutes and then washed twice with pure ethanol. Next, 10 uL of 600 Pa acrylamide mix (37.5 uL 40% acrylamide, 15 uL 2% Bis-acrylamide, 397 uL HCl solution (the amount of HCl is calibrated to obtain a final pH in the gel mix of 7), 10 uL of 500ug/mL NHS-acrylate in DMSO, 2 uL red fluorescent beads, 2.5 uL 10% APS, 0.25 uL TEMEDwere added on glass-bottom dishes and covered with a 13mm coverslip.

After gel polymerization (30 minutes), coverslips were removed and the gel was sterilized for 15 minutes using UV. Finally the gels were incubated for 2h at RT with 100 uL of 100 ug/mL Fibronectin solution.

Generation of chemoatractant gradients

SDF1 gradients will be generated using two diffusional methods: loaded heparin beads diffusion, or tissue secretion.

Measurement of intercellular forces

Simultaneously, cell-cell forces will be measured using monolayer stress microscopy (MSM) (15). MSM exploits the adhesion of cells onto a Fibronectin coated compliant polyacrylamide substrate where fluorescent tracker beads are embedded. Time-lapse imaging of the cell cluster and of the substrate underneath allows to keep tracking of the strains that the moving cluster will generate upon the substrate, therefore enabling high definition mapping of traction forces and stress transmission across the cluster during directed migration.

Calcium lowering

To interfere with cell-cell adhesions Calcium concentration in the medium is decreased by about 20%. The procedure requires the removal of 1 fifth (1mL) of standard medium and the supplement of Calcium-free DFA.

Pertubation of molecular mechanisms

Ultimately N-cadherin Morpholino perturbation in cluster motion will be used to acquire comprehension on the role in force sensing and transduction during Xenopus NCCs directed migration. Embryos are injected at 4-8 cell stage with 1 ng of Morpholino together with 10ng of FDX for labeling purpose.

Results

Gel Optimization

The first phase of substrate optimization required substantial modification of the gel preparation protocol. Our experience with cell lines leads us to under-estimate the importance of ECM cross-linker and ECM coating during the preparation phase. To obtain sustained cell adhesion, comparable with published results carried in plastic (14), a different strategy for ECM-crosslinker loading has been pursued: the NHS-acrylate molecule was loaded using a DMSO solution instead that ddH20. This allowed to accurately calculate the amount of crosslinker present in the gel, as NHS-arylate is totally soluble in DMSO only, and to perform comparable experiments. Moreover the

amount of Fibronectin loaded onto the gel was a 100ug/mL solution against a 15ug/mL used in previous works to provide sustained covalent binding. Implementing these modifications permitted to obtain optimal cell adhesion, which should occur in a lapse of 30 minutes in published works. This evidence clearly denotes a difference of primary cell culture against cell line culture, as the former has much less ability to secrete their own matrix compared with the latter.

Finally acrylamide percentages have been tested for force mapping purpose; with ranges of 600 to 3000 Pa. Traction force mapping was possible only in the softer (600Pa) condition (Fig.1).
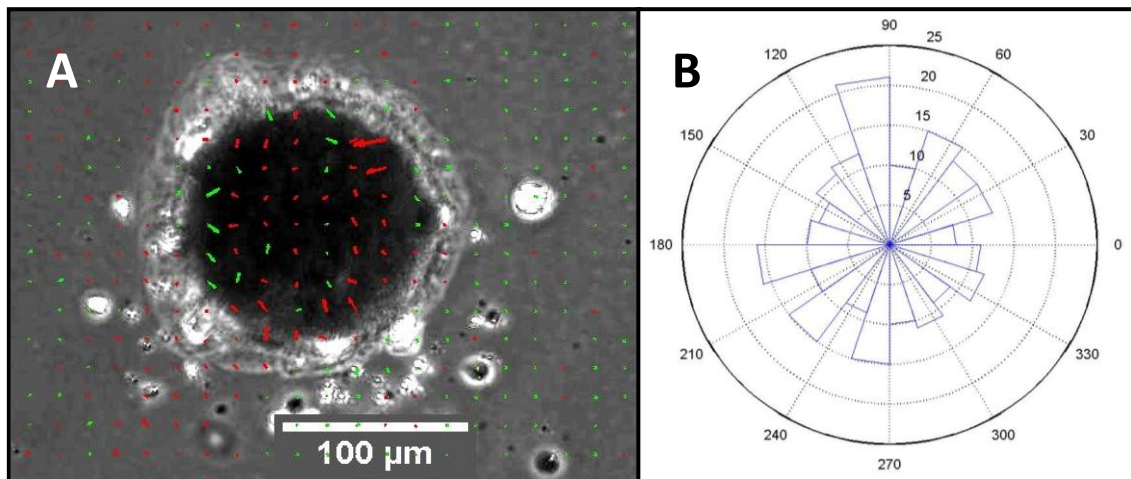

Fig.1 NC explants are able to adhere and generate measurable forces on a 600Pa PAA-gel (A, arrows represent force vectors, color coding the orientation, with vectors pointing to the left are in red and vectors pointing to the right are in green.). Orientation of the forces are randomly distributed (B).

Gradient Generation

The initial strategy pursued was based on previously tested method: Heparin Beads were incubated for one hour with high concentrations (e.g. 10ug/mL) of chemoattractant. Subsequently a silicon grease stripe was layered onto the gel and the loaded bead was pushed between the gel and the stripe. Cells showed to respond positively to a chemotactic gradient generated in such manner, but the technique readily presented an important limitation: the softness of the gel coupled with the distortions introduced by the stripe-bead system caused the introduction of a vast area (overlapped with the zone where cell migration occurred) of huge noise during force mapping.

As an alternative, we recognized that natural sources of soluble SDF1 are found within different tissues of *X.leavis* embryos. We started using cell generated chemotactic gradient by co-culturing NC explants together with Placode explant (stage 16) or Ectoderm explant (stage 10), both epithelial tissues, placed in close proximity (100-

200um) of the NC cluster. By this mean we obtained sustained directional migration of NC clusters toward a known source of chemoattractant that does not introduce problematic artifacts during force measurement (Fig. 2).
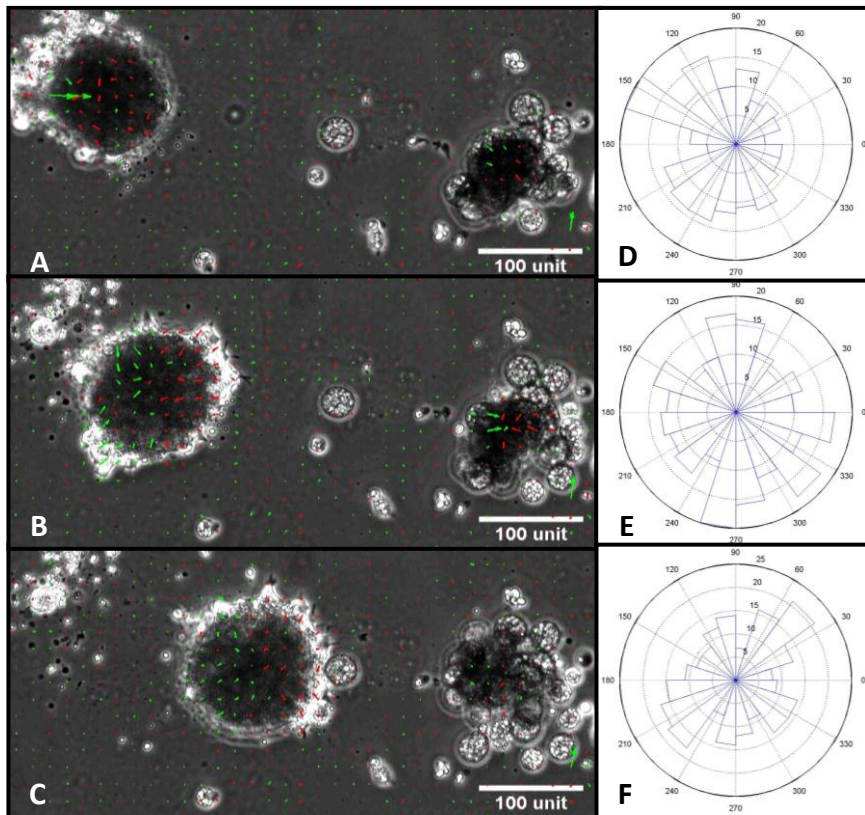

Fig.2 NC explant (A-C, left cluster) migrates chemotactically toward SDF1 secreting Placode explant (right) within at a speed of approximately 75um/h (every frame is taken every hour). Arrows represent force vectors, color coding the orientation, with vectors pointing to the left are in red and vectors pointing to the right are in green. D-F: traction forces are randomly distributed.

Traction Forces during chemotaxis

One very evident characteristic of traction forces inside the cluster is cooperativity. The force vectors of similar direction are clearly clustered together (Fig.3 A-C).
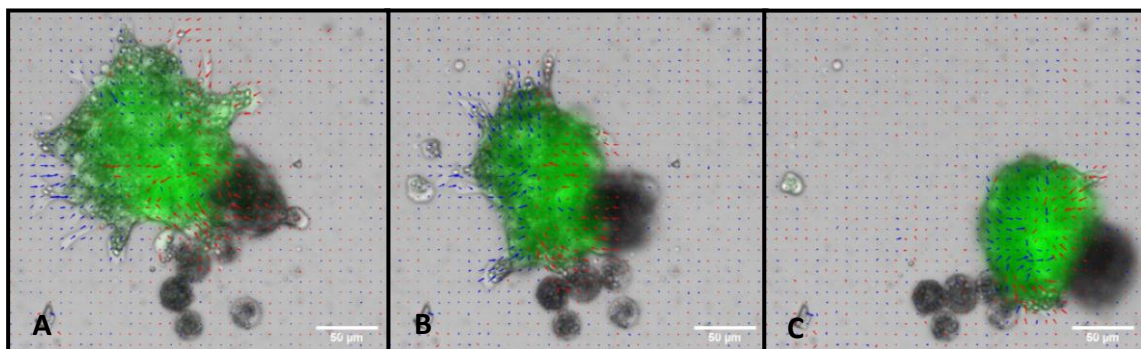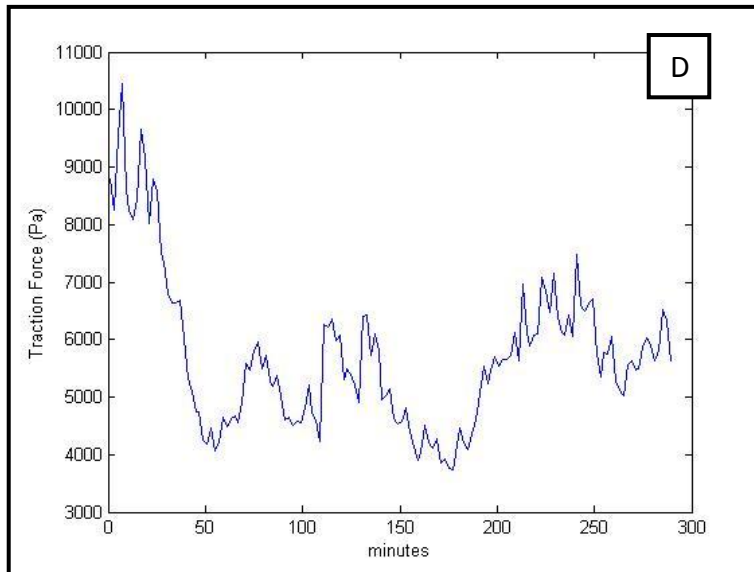
Fig. 3 A-C: during the first hour (reported images every 30 minutes) of time-lapse experiment the cluster of NC cells (green) migrate at a sustained speed toward the ectoderm explant, displacing it forward. The spreading of the NC explant is reduced concomitantly with this fast migration. During later image acquisition the speed was not so sustained (not shown). Arrows represent force vectors, color coding the orientation, with vectors pointing to the left are in red and vectors pointing to the right are in blue. D: it is interesting noticing the strong forces associated with the initial spread phase and the sudden reduction associated with the acceleration phase.

Perturbation of mechano-sensing molecules during directional migration

Our first molecular target was the surface molecule N-cadherin, because of its central role in developing cell-cell contact in the NC tissue and because it also play a role in the molecular regulation of collective behavior during migration of this tissue (14).

We selected 3 basic strategies to inhibit its functions: 1) inhibit its Calcium-dependent homo-dimerization across the extracellular space by reducing the Ca2+ concentration in the medium in the range of 20% (published method, 14); 2) knocking down the mRNA expression by injecting the correspondent Morpholino during early (8 cell stage) embryo formation.

1) Our early results show that a decrease of 20% of Ca2+ in the culturing medium triggers a fast dispersion of the cells present in the initial cluster, interrupting the chemotaxis (Fig. 4). A strong diminishment of traction forces is evident, leading to speculate that the mechanical connection operated by N-cad is also important to stimulate the generation of forces onto the substrate, depositing in favor the relevance of N-cad in maintaining the cooperative behavior of

directed migration. Unfortunately the disaggregation of the cluster is so fast that it's hard to define refined dynamics during the loss of cooperativity.
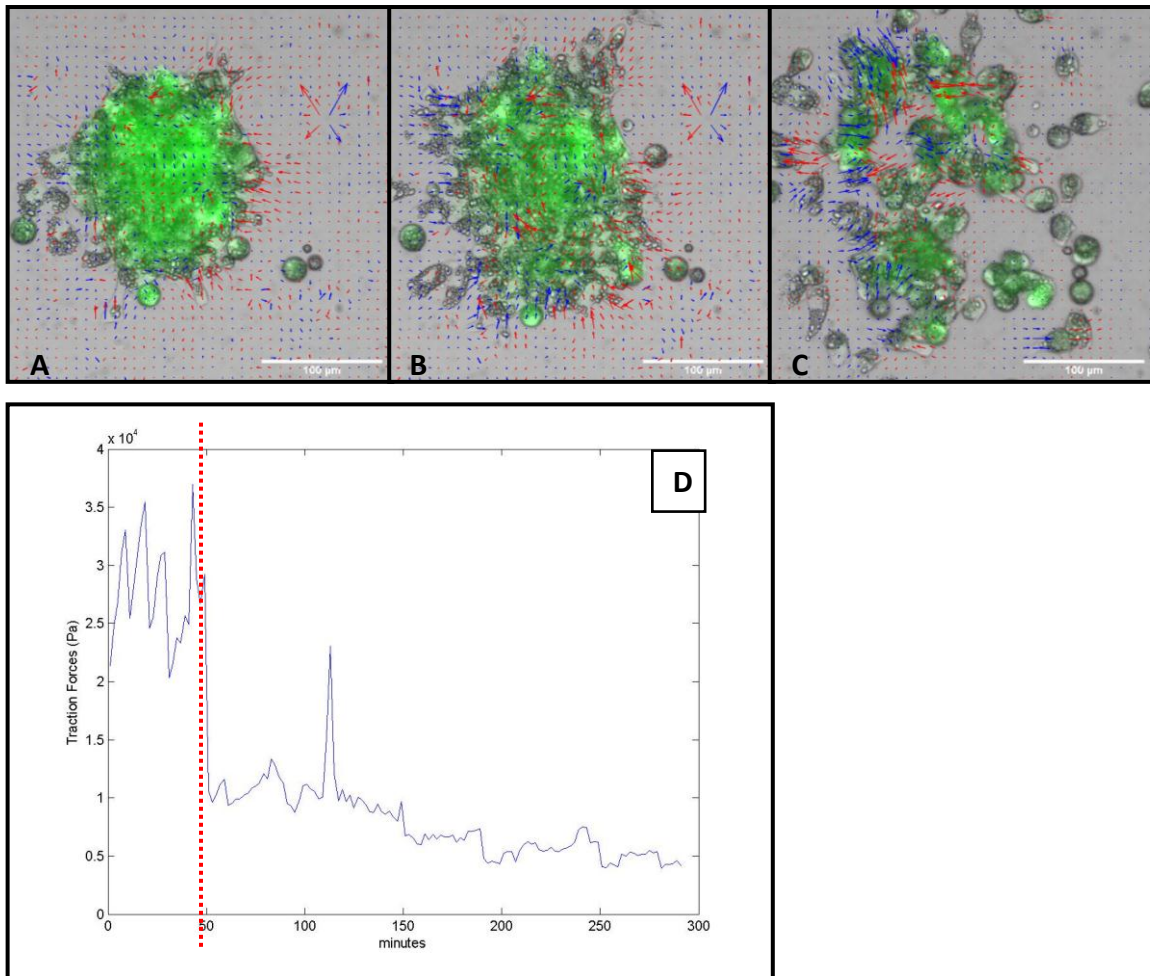

Fig. 4 A-C: images every 50 minutes, low Ca2+ medium introduced after 50 minutes, cluster dispersed within 30-50 minutes. Arrows represent force vectors, color coding the orientation, with vectors pointing to the left are in red and vectors pointing to the right are in blue. D: graphic showing the strong decrease in traction force after the reduction of Ca2+ concentration (dashed red line).

2) Previous experiments using N-cad Morpholino (14) showed a similar disruption of cluster integrity of that induced by lowering Ca2+ in the culturing medium. Using the standard concentration (1ng/nL) of Morpholino it was not clear that the treatment could reproduce the effect previously described. We couldn't observe comparable dispersion, although the morphant explant seems to lose the cooperative characteristic, showing a more irregular morphology (Fig. 5).
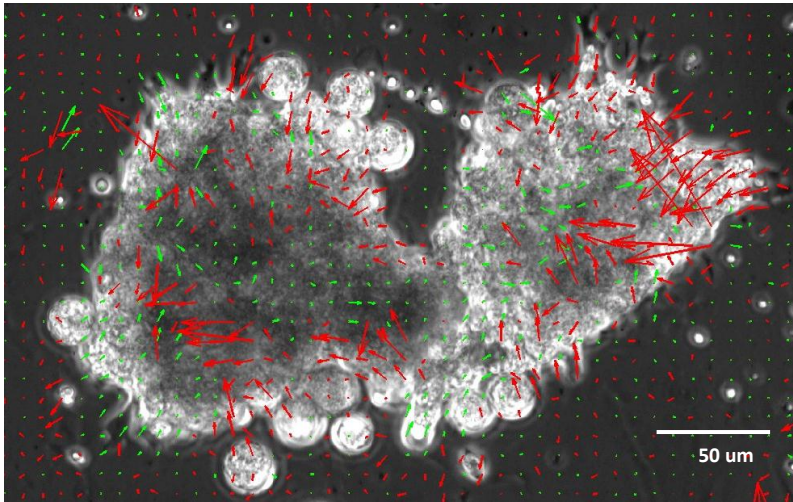
Fig. 5 Neural Crest Explant transfected with N-cadherin Morpholino shows irregular morphology but fails to completely dissociate. Arrows represent force vectors, color coding the orientation, with vectors pointing to the left are in red and vectors pointing to the right are in green.

It was often observed a sorting phenomenon: not all the NC cells received the same amount of Morpholino (distinguished by absence of FDX staining), in which case they were able to move chemotactically toward an ectoderm explant. (Fig. 6)

This series of experiment brought to light an unexpected observation: after about 5 hours of time-lapse the ectoderm tissue placed in proximity of NC cluster as chemotactic source experienced a differentiation event, manifested as increased spreading and traction forces, coupled with chemotactic movement toward the NC explant (Fig. 7). This is a very new finding that we are currently characterizing: on Fibronectin coated gels Ectoderm adhesion is severely delayed, taking at least 5 hours for the explant to successfully spread and start migration. This delay in adhesion prevented us from noticing the chemotactic movement of the ectoderm until we did not impair the correct movement of NC cells. We are currently searching a strategy to improve the chemotactic movement of Ectoderm and impair the migration in NC explants.
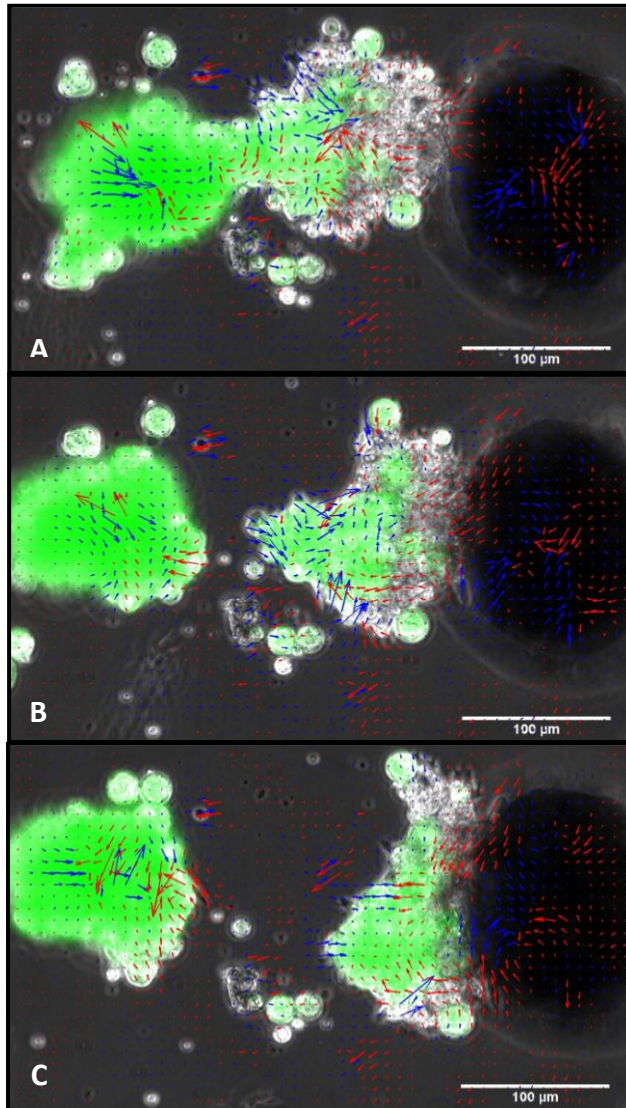
Fig. 6 A-C: images displaying time-lapse frames taken every 15 minutes. Neural crest explant (green) is in process of sorting, with a part presenting an area of non-transfected cells (missing green staining) that are able to undergo a strong chemotactic migration, and another area that doesn't respond to the presence of chemotactic source. Arrows represent force vectors, color coding the orientation, with vectors pointing to the left are in red and vectors pointing to the right are in blue.

Conclusion and Future Work

Our work aims at defining the role of traction forces exerted by a coherent cluster of specialized migratory cell during chemotaxis. It has been possible to develop an efficient framework that allows studying traction forces by the use of an optimized poly-acrylamide gel coupled with a functional strategy to generate a chemotactic gradient onto it. The gradient can be generated by artificial mean (heparin bead release system) or by co-culturing the model tissue (Neural Crest in *X.leavis*) together with an endogenous chemoattractant gradient generating tissue, like Placode or Ectoderm. Although both strategies are able to elicit the same response by the NC

cells, only by using and endogenous tissue it was possible to avoid artifacts, since the heparin bead strategy introduced enormous deformation on the gel surface.
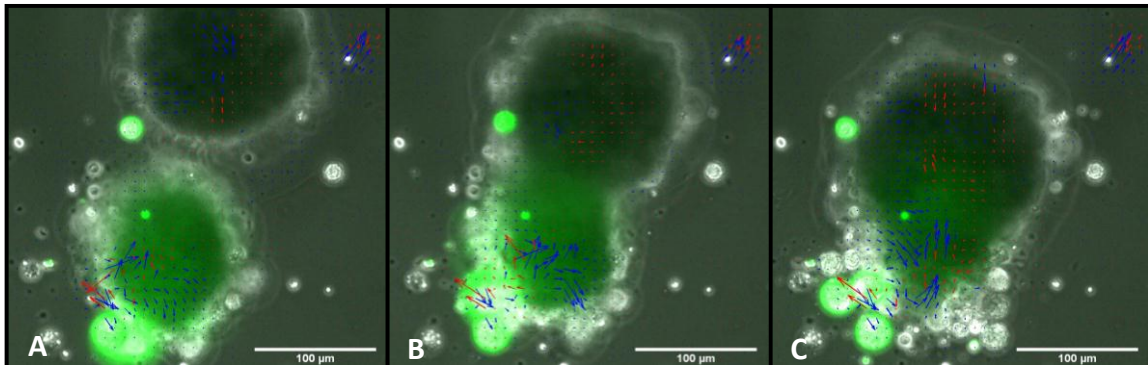

Fig. 7 A-C: images displaying time-lapse frames taken every 20 minutes. NC explant transfected with N-cadherin Morpholino (green) are relatively immotile compared with Ectoderm explant, which promptly spread onto the substrate and start directed migration toward the NC cluster. Arrows represent force vectors, color coding the orientation, with vectors pointing to the left are in red and vectors pointing to the right are in blue.

In order to migrate in a chemotactic fashion the cells in the NC explants need to be in mechanical connection by means of N-cadherin molecules involved in efficient cell polarization establishment. It has already been shown that disruption of these junctions seriously impairs chemotaxis. Our work shows that N-cadherin is not only involved at the biochemical level, regulating cell polarization, but ultimately is generating an order in the traction force directions. Our traction maps shows clear zoning of traction force vectors in control condition. It is clear indeed that tractions generated on one edge of a cluster are transmitted toward the center, and traction forces propagated from the opposite edge have a specular vector component. It is also clear that when normal N-cadherin functionality is impaired (by Morpholino down-regulation) traction forces are less zoned compared with wild type condition, accompanying the reduction of regularity of cluster morphology. When functional N-cadherin is normally expressed but its homo-dimerization at the cell-cell junction is impaired by extracellular Ca2+ level decrease prompt cluster dispersion is triggered and forces obviously reduce their zoning together with their total intensity. This seems suggesting that efficient mechanical connection between cells favors their "fitness", or better said their ability to generate traction forces, thus N-cadherin based cell-cell junctions could positively influence cell contractility in a cluster by a mechanism that yet has to be elucidated.

During the experiments we could observe a phenomenon never described before: by impairing NC chemotaxis by down-regulating N-cadherin we could observe a late chemotactic response of Ectoderm tissue toward the NC cluster. It is known that NC cells secrete themselves a chemokine: C3a (Complex fragment 3a, ref. 16), that they use paracrinaly to maintain the integrity of the cluster. Evidences obtained in parallel studies shown that ectoderm cells potentially express the receptor for this molecule (unpublished datas) and therefore are able to respond to a C3a gradient. This is likely to be the first time that an epithelial tissue could be used as a collective chemotaxis model. During ectoderm chemotactic migration traction force maps have been generated, and apparently the forces exerted by this tissue are less coherent compared with those generated by NCs, but more experiments are needed to establish it.

Taken together, the informations disclosed in the present report have the potentiality to improve our knowledge on fundamental migration processes observed during many different biological events, both in physiological (embryogenesis, wound healing) and pathological (cancer) conditions, since they provide an insight on the connections between the biochemical and the mechanical regulations operating inside migrating cell clusters.

The study is not yet completed, as we still haven't assessed the influence of other proteins (possibly involved in mechano-sensing processes) such as alpha and beta catenin, vinculin, and p120 and their influence on chemotaxis and traction force orchestration. Moreover, considering the novelty of the experimental framework, we urgently need to develop powerful algorithms for traction force analysis based on experimental observations of this very relevant model.

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