Scientific report – Short visit grant 4882
1) Purpose of the visit.

Quantitative analysis of cytoskeletal dynamics during morphogenesis

The purpose of the visit was to develop further an ongoing collaboration between my lab and Alfonso Martinez Arias lab on the quantitative understanding of morphogenesis using Dorsal Closure in Drosophila as a model system. In the last years, Dorsal Closure (DC) has emerged as a reference model system where to study dynamic morphogenetic processes using quantitative and biophysical approaches (reviewed in (Gorfinkiel et al., 2011)). DC is a morphogenetic process that takes place during Drosophila embryogenesis, by which interactions between two tissues, the amnioserosa (AS) and the epidermis, close a discontinuity at the dorsal side of the embryo and generate the final form of the larvae (reviewed in (Jacinto et al., 2002). The work in my lab in Madrid is devoted to the quantitative analysis of cell behaviour during this process and for this we use live imaging of embryos carrying different fluorescent reporters (membrane, cytoskeletal, adhesion), custom-developed software to quantitatively analyze different features of cell behaviour, genetic tools to abolish or over-activate specific molecular components and laser ablation to mechanically perturb the system. The work in Alfonso Martinez Arias lab, lead by Pedro F. Machado, is focused on the development of a theoretical framework in which to analyze the mechanochemical coupling underlying cell and tissue behaviour.

Apical cell contraction is a cell shape change recurrently used during morphogenesis that is at the basis of essential processes such as gastrulation, neural tube formation and wound healing (Schock and Perrimon, 2002). During DC, AS cells contract their apical surface area thus generating one of the major forces driving closure (Kiehart et al., 2000; Gorfinkiel et al., 2009). One of the most important observations in the last few years on the dynamic behaviour of apically contracting cells is that cells do not reduce their apical surface continuously but rather undergo cyclic phases of contraction and expansion driven by the transient formation of actomyosin foci that flow accross the apical region of cells (reviewed in (Gorfinkiel and Blanchard, 2011)). We have observed that the frequency and amplitude of these oscillatory area fluctuations in the AS exhibit spatial and temporal patterns as the whole tissue contracts (Blanchard et al., 2010). Also, we have detected a strong positive correlation between cell area fluctuation frequency and rate of contraction. Collectively, these results suggest that cell fluctuations play an important role in the behaviour of the tissue and the integration of the associated deforming forces.

Two main questions arise from these observations. The first refers to the nature of the oscillatory behaviour: what is the mechanism underlying the periodic assembling and disassembling of actomyosin foci, and whether the oscillations are cell autonomous or emerge from the intercellular coupling that gives rise to tissues. The second one refers to the mechanism of apical cell contraction, or how do cells transform the fluctuating behaviour into an effective contraction. It has been suggested the existence of a ratchet mechanism that would prevent the relaxation phase of fluctuations and would thus maintain the contracted state of cells. Both the existence of internal and external racthets have been postulated although our results support that in the AS the main ratcheting mechanism is intracellular (Martin et al., 2009; Solon et al., 2009; Blanchard et al., 2010).

Pedro and I have started a multi-disciplinary collaborative project two years ago to tackle these questions. During this time, Pedro has developed a minimal theoretical model of AS cell fluctuations that combines cell elasticty, contractility and cortex turnover following the approach used in (Sedzinski et al., 2011). The main attributes of the model are the following:

-cells are considered passive viscoelastic materials,

-active forces are contractile and myosin driven,

-changes in actin density are due to the intrinsic turnover of the actin cortex and to the change in cell surface area, since actin density is increased when the area is reduced.

-changes in myosin density follow changes in actin density.

These essential features can be formulated in a system of 3 differential equations that describe: 1) the change in surface area (ds/dt) resulting from the sum of myosin driven contractile forces and passive viscoelastic forces exerted at the level of radial struts and cell edges respectively, 2) the change in actin concentration (dA/dt) resulting from the active turnover of the actin cortex (rates of actin polymerisation and depolymerisation) and to the changes in cell area, and 3) the change in myosin density (dm/dt) results from the on/off rates of myosin onto actin filaments.

A key component of this system for oscillations to occur is the presence of two characteristic timescales: the viscoelastic relaxation timescale for the change in area given by the viscoelastic properties of the cell and the actin cortex turonover timescale, which are coupled in the following equation for actin dynamics:

$$\frac{dA}{dt} = \frac{1}{\tau}(Sa' - A) \qquad \text{(Eq. 1)}$$

where τ is the actin turnover time, a' is an equilibrium actin density , A is actin concentration and S is cell area.

Assuming that myosin on/off rates are much faster than polymerisation/depolymerisation rates (kon,koff>>kp,kd), changes in myosin can be assimilated to changes in actin (dm~da) and thus the system is reduced to 2 equations with 5 free parameters. By rescaling length, time, and densities, we are left with two essential parameters:
-ε, the ratio between the viscoelastic relaxation timescale and the actin turnover timescale
-μ, the ratio between the active forces (myosin driven) and the passive forces (viscoelastic resistance).

It is then possible to search for the region of the parameter space where this system of two equations can generate autonomous, sustained oscillations with amplitudes and frequencies that match the experimental data (Blanchard et al, 2010). Because we have observed that these properties evolve as DC progresses it is expected that the model parameters, ε and μ, will also evolve over time. In particular, the model predicts that ε increases and μ decreases over time. Both our unpublished work (Pedro F. Machado and N. Gorfinkiel, unpublished) and published work on the mechanical properties of AS cells using laser ablation (Ma et al., 2009) suggest that the AS evolves from a more fluid-like to a more solid-like material, i. e. the viscoelastic relaxation timescale decreases. Because ε is the ratio of viscoelastic relaxation to actin polymerisation/depolymerisation timescales, the model thus also predicts that actin polymerisation/depolymerisation rates have also to decrease.

The other main feature of the model is that in order to reproduce the progressive contraction of cells some kind of ratchet, that prevents the relaxation of a cell's area after a round of contraction, has to be implemented. In the AS, two possible scenarios have been postulated. While the work form Solon et al, 2009, suggests the existence of an extra-cellular ratchet provided by the supra-cellular actin cable that forms at the interface of the AS and the epidermis, our work provide evidence for the existence of an intra-cellular ratchet operating at the level of individual cells (Blanchard et al., 2010). At the moment, the nature of such intra-cellular ratchet remains elusive. Thus, it is important to explore this issue experimentally to feed the model with the more realistic approach.

During my stay in Cambridge, we have focused on these two aspects of the model related to the actin cortex turnover rate and ratchet mechanism. Through quantitative image analysis, we searched to validate the prediction of the model concerning the evolution of the actin cortex turnover rate and we explored the nature of the ratchet mechanism.

2) Description of the work carried out during the visit and main results obtained.

2.1) Measurement of the actin cortex turnover rate

To be able to measure the actin cortex turnover rate, in my lab we have generated high time resolution time-lapses of embryos carrying a membrane marker (ArmYFP), an actin reporter (the actin binding domain of the protein moesin bound to GFP –sGMCA), and a myosin reporter (the myosin regulatory light chain fused to the fluorescent protein Cherry –mCherry), during early, slow and fast phases of DC. During my stay in Cambridge I have analyzed these movies using software developed by Richard Adams and Guy Blanchard, from the Departement of Physiology. Development and Neuroscience, and obtained quantitative data on the area and the actin and myosin fluorescence. This data can then be fitted to the equation of the model describing changes in actin density (Eq. 1).

Our goal is to estimate the parameters τ and a'. Taking S and A to be independent variables that can be extracted from the ArmYFPsGMCAmCherry movies and using Equation 1, it is possible to perform a multilinear regression of the form:

$$y = \beta' S + \beta'' A + \epsilon \qquad \text{(Eq. 2)}$$

where $y = \frac{dA}{dt}, \beta' = \frac{\alpha'}{\tau}, \beta'' = -\frac{1}{\tau}$ and ϵ is the residual error.

ArmYFPsGMCAmCherry movies give us raw data in terms of *A(t)* and *S(t)* at every time frame t. In order to reduce the high-frequency noise arising from the imaging and segmentation process, our first step was to perform a 5-step moving average on *A(t)* and *S(t)* such that, e.g.,

$$A(t) = \frac{1}{5}\big(A(t-2) + A(t-1) + A(t) + A(t+1) + A(t+2)\big)$$

In order to compute $\frac{dA}{dt}$ we then calculated the central finite differenceof *A(t)* to second order in Δt, e.g.,

$$\frac{dA}{dt} \approx \frac{1}{2\Delta t \Delta t}(A(t+1) - A(t-1))$$

where Δt is the time interval between consecutive time frames. The end result is filtered time-series data of the form $\{\frac{dA}{dt}, A(t), S(t)\}$ for every cell and embryo. 3D scatterplots of this data for a sample embryo are shown in Fig.1.

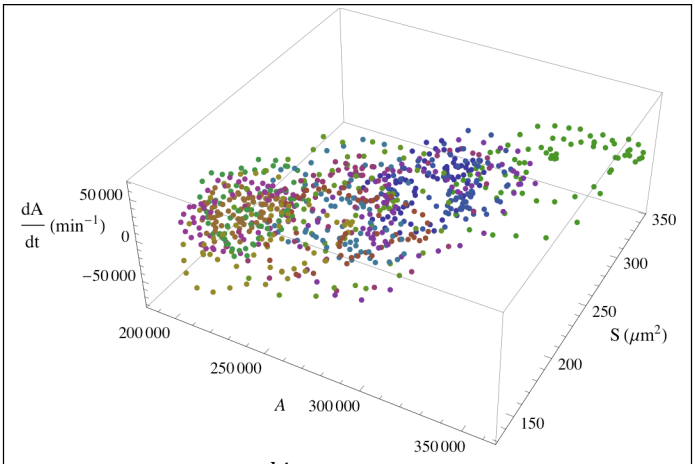

Fig. 1. 3D scatterplot of $\frac{dA}{dt}, A(t), S(t)$.

We performed a multilinear regression analysis of the form shown in Eq. 2 using the filtered time-series data to extract the parameter estimates for α' and $\frac{1}{\tau}$ for each cell and embryo. The distribution of the cell means of these estimates as well as the mean estimate for each embryo are shown in Fig. 2A, B, C. If we pool the embryo data according to stage (early and slow DC embryos), we get the mean α' and $\frac{1}{\tau}$ for the early and slow phase of DC (Fig. 2D). These results show that reference actin density α' is the same for both early and slow DC phases. In contrast, the actin turnover $\frac{1}{\tau}$ differs such that τ decreases, in agreement with the model predictions.

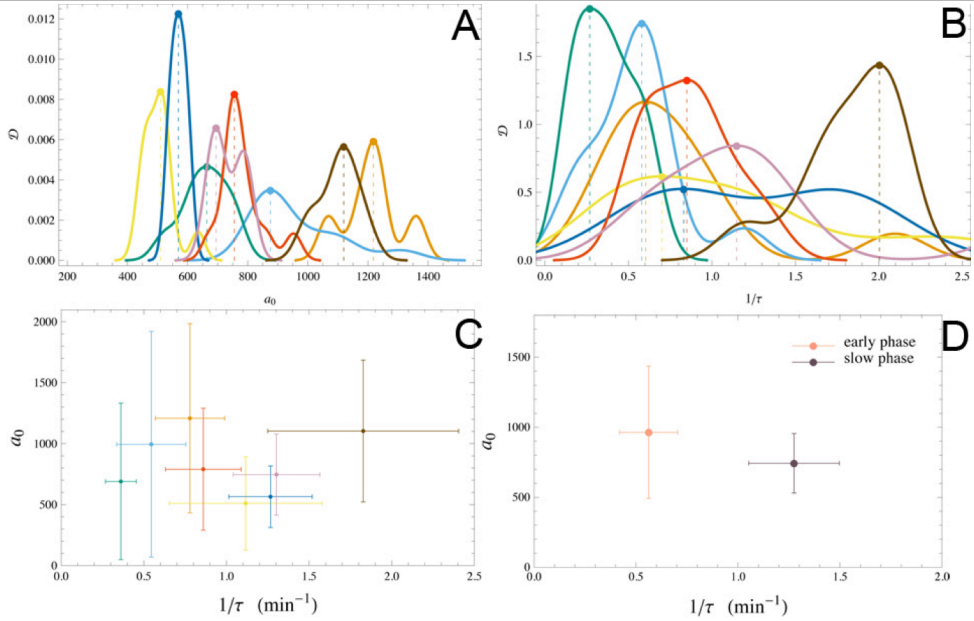

Fig. 2. (A, B) Probability density of the cell parameters α' (A) and $\frac{1}{\tau}$ (B) per embryo (each colour line is a different embryo). (C) Mean α' and $\frac{1}{\tau}$ for the different embryos analyzed. (D) Mean α' and $\frac{1}{\tau}$ for different DC stages. Performing a two-factor ANOVA on the parameters shows that $\frac{1}{\tau}$ is significantly dependent on the DC stage (p=0.00823).

2.2) Exploring the nature of the ratchet mechanism underlying AS cell apical contraction

Our previous work has shown that myosin is localized in AS cells in two populations: at the medial region as dynamic foci and at the level of cell-cell junctions (Blanchard et al., 2010), and that both of them increase in density over time. An attractive hypothesis is that medial myosin foci drive cell area oscillations whereas junctional myosin provides the ratcheting mechanism (Blanchard et al., 2010; Gorfinkiel and Blanchard, 2011).

In order to explore this possibility, in my lab, we have generated time lapse movies of DC embryos carrying a membrane marker (DECadherin-mTomato) and a myosin reporter (a Yellow Fluorescent Protein inserted in the myosin heavy chain *–zipper-* locus), with the idea to measure how myosin fluorescence intensity at the medial and junctional regions evolve over time. The specific question we asked is whether there is a change in the relative contribution of medial and junctional Myosin as DC progresses.

I analyzed these movies in Cambridge using the software mentioned previously. The tracking itself was quite challenging since the membrane marker bleaches over time and the signal drops to levels that cannot be detected reliably by the tracking software. To overcome this, Guy Blanchard has implemented a manual tracking tool which greatly ameliorates the segmentation and tracking efficiency. Once the segmentation is done, several processes, which require heavy quality checks by the user, have to be run to obtain basic information on cell metrics such as cell area, cell perimeter, rate of area change, etc. The algorithms to extract quantitative data on fluorescence intensity have also evolved over time as part of my long term collaboration with Guy Blanchard.

Total and average (~ density) fluorescence intensity per cell at the medial region and at the level of cell junctions can then be obtained and its dynamics over time analyzed. The first observation that comes up from these analysis is that although medial and junctional densities increase over time (Fig. 3A, A'), total medial and junctional myosin fluorescence decrease (Fig. 3B, B').

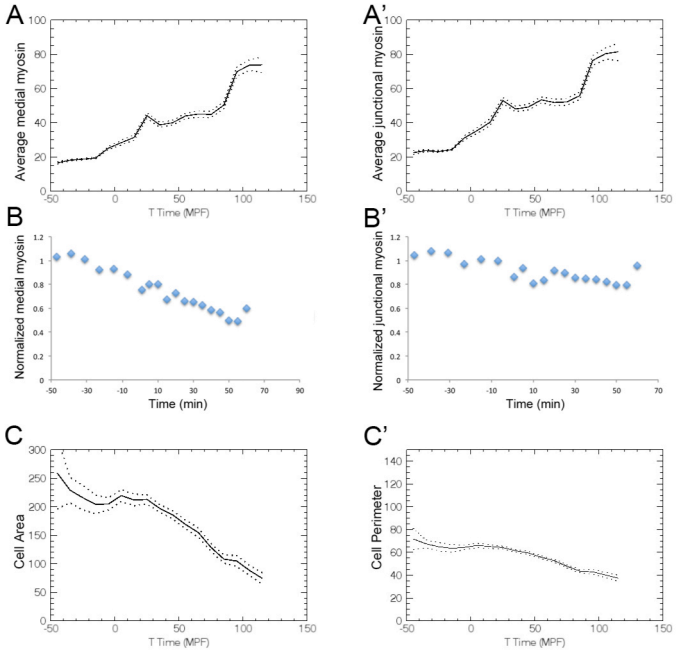

Fig. 3. Medial (A,B) and junctional (A', B') myosin fluorescence intensity levels during DC. While average myosin levels increase (A, A'), total myosin levels decrease (B, B'). As cells contract, apical cell surface area (C) and cell perimeter (C') also decrease.

These results suggest that as cells contract they have to remove their contractile machinery but they seem to do so at a different rate than the cell area and cell perimeter contract (Fig. 3C, C'), otherwise the density of the two myosin populations would remain constant.The question that then arises is how the levels of medial and junctional myosin intensity decrease compared to the rate of cell area and cell perimeter decrease.

To analyze this, we calculated two parameters: the ratio of junctional to medial myosin (R_1) and the ratio of cell perimeter to cell area (R_2). We reasoned that if medial and junctional myosin were decreasing passively, just because the cell area and cell perimeter were decreasing, the ratio between R_1 and R_2 would remain constant during the process. In contrast, if there was an active upregulation of medial or junctional myosin, the ratio between R_1 and R_2 would decrease or increase, respectively (Fig. 4A).

Interestingly, we found that the ratio between R_1 and R_2 increases in 6 out of 7 embryos analyzed (Fig. 4B), suggesting that during DC, there is an active recruitment or stabilization of junctional myosin compared to medial myosin. We think this result is very interesting and may provide preliminar evidence of the existence of a junctional ratchet driven by the preferential accumulation of myosin at the level of cell-cell contacts that could stiffen the membranes and could thus provide a ratcheting mechanism.

These results are leading Pedro to implement a junctional ratchet by increasing myosin levels at the level of cell-cell junctions at a constant rate while myosin levels at the level of radial spokes decreases coordinately.

In summary, the work developed by Pedro F. Machado and myslef at the University of Cambridge has validated some of the predictions of the model and has suggested ways to implement the ratcheting mechanism required to simulate the contraction of the cells.

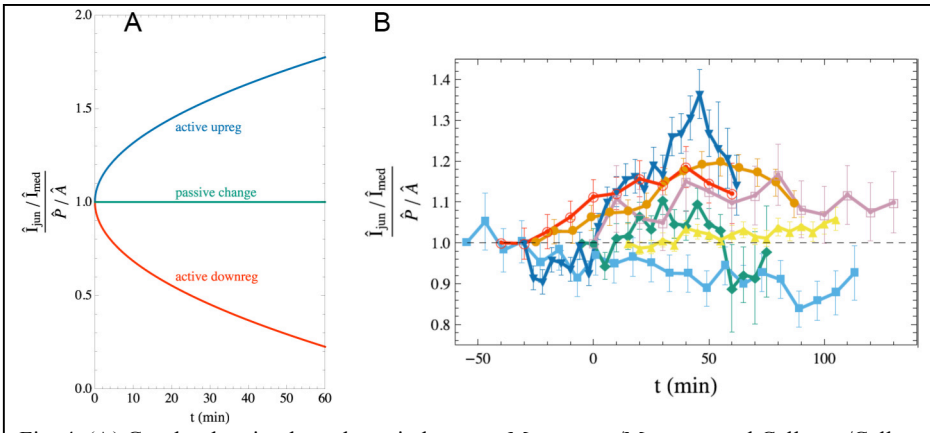

Fig. 4. (A) Graphs showing how the ratio between $Myo_{junctional}/Myo_{medial}$ and Cell area/Cell perimeter would evolve if the decrease in both myosin populations were passive (green line), there were a passive downregulation of junctional myosin relative to medial myosin (red line) or there were an active upregulation of junctional myosin relative to medial myosin (blue line). (B) Data from 7 embryos showing in all except one an active upregulation of junctional myosin relative to medial myosin.

3) Future collaboration with host institution

The work developed during my stay in Cambridge supported by the QuanTissue grant is part of a long term collaboration between my lab and Alfonso Martinez Arias' lab. Although my stay in Cambridge was very important to strengthen the interplay between experiments and theory, there are several issues that need more work. In particular, more data is required to have better values for the evolution of the actin turnover rate during DC. Moreover, Pedro is developing the way to also extract information on the myosin on/off rates from these movies, that the model predicts are much faster than the actin de/polymerisation rates. Related to the ratchet, in my lab we will generate more time lapses in which to measure the evolution of medial and junctional myosin as well as movies that will allow to measure medial and junctional actin populations. We will also search for ways to perturb independently junctional and medial myosin. Finally, we are thinking of performing FRAP experiments of medial and junctional myosin to obtain independent measures of myosin turnover rate.

4) Projected publications to result from the grant

We are planning to start writing a mansucript describing the biophysical model of DC developed b Pedro F. Machado with the experimental validations mentioned above and the ESF and the QuanTissue program will be acknowledged as specified in the Grant guidelines.

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