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Reaction–diffusion microtubule concentration patterns occur during biological morphogenesis

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Abstract

Reaction–diffusion processes can lead to a macroscopic concentration pattern from an initially homogeneous solution, and thus provide a physical–chemical mechanism for biological pattern formation and morphogenesis. The central prediction of reaction–diffusion theory is that the patterns contain periodic concentration variations in some of the reactives. Microtubules assembled in vitro spontaneously self-organise and form stationary striped macroscopic structures. In agreement with reaction–diffusion theory. Here we show, in agreement with reaction–diffusion theory, that these preparations contain substantial microtubule concentration variations. Similar striped microtubule patterns arise during *Drosophila* embryogenesis. A characteristic of these patterns is their dependence on sample dimensions. In *Drosophila* eggs shortened by ligation, we found that the microtubule pattern varied with egg fragment length in the same way as the in vitro microtubule pattern varied with sample length, and as expected from theory. This is evidence that reaction–diffusion structures occur during *Drosophila* morphogenesis.

Keywords: Biological self-organisation; Reaction–diffusion structures; Dissipative structures; Microtubules; *Drosophila* embryogenesis

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Theoreticians [1–3] have predicted that reaction–diffusion processes in specific types of chemical reactions that are far-from-equilibrium and contain an auto-catalytic step, might lead to the formation of a macroscopically self-organised chemical pattern from an initially homogeneous solution. Chemical energy is dissipated and a stationary macroscopic pattern, made up of variations in the concentration of some of the reactives, progressively develops. Turing in 1952 [1] proposed that such mechanisms might provide a physical–chemical basis for biological morphogenesis. However, it was not until 1990 that a variation of the Belouzov–Zhabotinsky reaction was accepted as the first experimental example of such a structure [42]. Although numerous comparisons of biological morphologies with the predictions of reaction–diffusion theory have been made [24–29], there are no in vitro examples of biochemical systems behaving this way. Under suitable conditions, the formation of microtubules from tubulin self-organise in the manner expected for a reaction–diffusion system.

Microtubules [18,19] are tubular shaped entities, 240 Å in diameter and several microns long, made from tubulin dimers. They may be formed in vitro by warming a cold solution of purified tubulin to 30–35°C, in the presence of guanosine triphosphate (GTP). Chemical reactions occur, GTP is hydrolysed to guanosine diphosphate (GDP), and within a few minutes the tubulin assembles into microtubules. After initial microtubule assembly, GTP hydrolysis continues by a process in which tubulin is added to one end of microtubules whilst being lost from the other [20].

The assembly process has an auto-catalytic step and can show non-linear kinetics [21]. Under suitable conditions, initially homogeneous solutions of tubulin form microtubule solutions that progressively self-organise over 5–6 h to form stationary macroscopic patterns that remain stable for approximately 3 days [4–7]. The patterns are readily observed with polarisation optics, and the blue and yellow stripes of 0.5-mm separation shown in Figs. 1 and 3 arise from microtubule orientations that alternate periodically from acute to obtuse. Each stripe is itself comprised of stripes of 100-μm separation, and these small stripes in turn contain 20-μm separation stripes.

Homogeneous solution of chemicals and biochemicals do not normally self-organise to form macroscopic concentration patterns, and the fundamental physical–chemical processes underlying the formation of biological concentration patterns remain unknown. In biological morphogenesis, positional information is associated with concentration patterns of various substances [22,23]. It has been proposed that they might initially arise from reaction–diffusion processes [1–3,24–29]. As the central prediction of reaction–diffusion theory is the formation of macroscopic concentration patterns from an initially homogeneous solution we wanted to know whether the self-organised microtubule structure contained periodic microtubule concentration variations. Experimentally, this is complicated by the pattern of orientational variations present. In this report we used two independent methods, small angle neutron scattering and fluorescent imaging, to show the presence of microtubule concentration variations of approximately 25% of the average. Thus, in agreement with the main prediction of reaction–diffusion theory, a periodic pattern of microtubule concentration exists in the solution.

Neutron small angle scattering from anisotropic objects in solution, depends upon the shape, dimensions, concentration, and orientation of the scatterers [30]. Shape and size information is contained in the dependence of the scattered intensity with radial angle. The orientational information is in the azimuthal angular distribution. If, as in the present case, the solution is dilute and orientations cut the Ewald sphere, the radial average of the scattered intensity is proportional to their concentration [31]. We measured the small angle neutron scattering[1] from self-organised microtubule preparations made up in spectrophotometer cells ($40 \times 10 \times 1$ mm), and showing

[1]Small angle neutron scattering measurement were carried out at the Institut Laüe Langevin (Grenoble) using the D22 camera, with 10-Å wavelength neutrons and a sample to detector distance of 2.5 m. The range of reciprocal space covered was from 0.01 to 0.12 $Å^{-1}$.

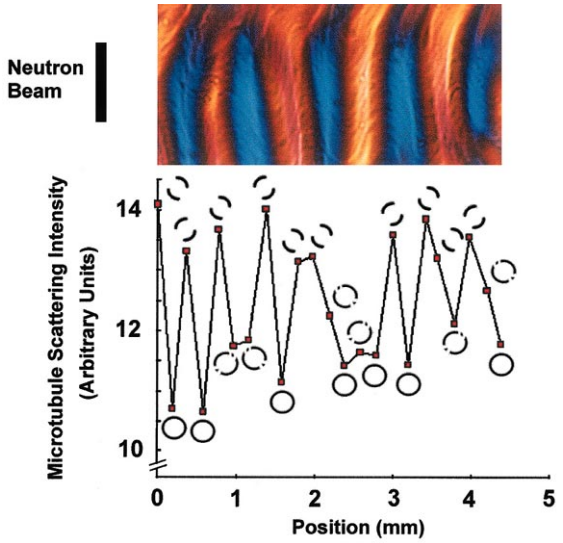

Fig. 1. Microtubule concentration patterns as shown by small angle neutron scattering. The upper photograph shows part of a self-organised microtubule preparation initially containing 10 mg/ml of tubulin [4–7] was photographed through crossed linear polars with a wavelength retardation plate at 45°. The blue and yellow interference colours arise from microtubule orientations that are respectively acute and obtuse to the long axis of the sample cell. Neutron small angle scattering spectra were recorded every 30 min. The beam measuring 0.15×1 mm was progressively stepped (0.2 mm) along the long axis of the cell. The scattered intensity as a function of reciprocal space gave the known microtubule scattering curve [4–7,40]. The variation in the microtubule scattering intensity with position shows periodic variations in microtubule concentration of approximately 25%. The icons show the intensity distribution on the two dimensional detector, and indicate the microtubule orientation.

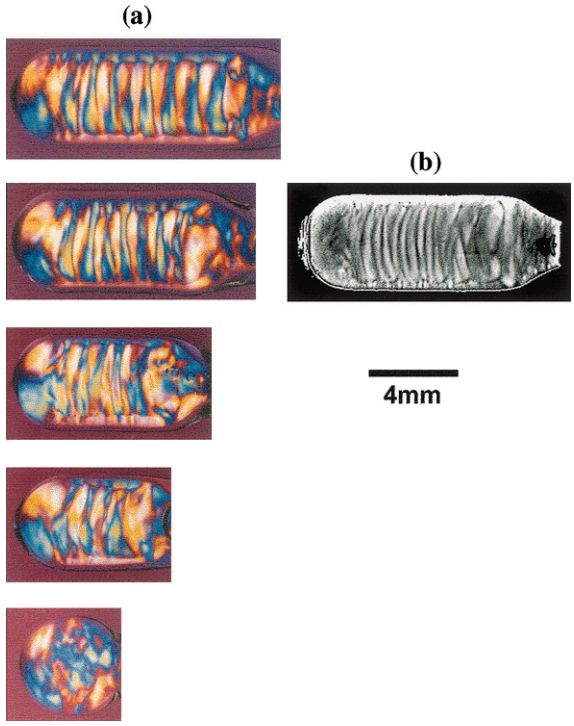

Fig. 3. Effect of sample length on microtubules patterns. (a) Birefringent patterns formed by microtubules [4–7] assembled in cylindrical 'egg' shaped sample containers. There are two stripe free regions at each end of the sample, separated by a striped central zone. Below a certain critical sample length, the striped central region does not form. For longer samples, the end zones remain of the same length, and the number of stripes in the central region increase with sample length. This type of behaviour agrees with theoretical predictions for certain types of reaction–diffusion systems. (b) Microtubule concentration variations as detected by DAPI fluorecence in a cylindrical container. The overall morphology resembles the microtubule pattern in *Drosophila* eggs.

stripes of approximately 0.5-mm separation. The dependence of the scattered intensity on radial angle (2θ) or more precisely momentum transfer (Q) yield a series of secondary maxima due to the tubular shape of the microtubules. Analysis of this pattern gives values of the inner and outer radii in agreement with the known values for microtubules [40,41]. In addition, the scattering is concentrated into arcs, which depending on the stripe examined, are at either approximately 45° or 135° to the horizontal. The sample was examined with a small neutron beam, 0.15×1 mm, and the microtubule scattering recorded at different positions in the sample. Microtubule concentration as a function of position was determined by measuring the microtubule scattering, in steps of 0.2 mm, along a direction perpendicular to the stripes. The changes in microtubule orientation detected in the neutron measurements coincide with those seen optically. The scattering intensity varied periodically with position. Variations of microtubule concentration are observed; the concentration drops by approximately 25% when the orientation changes from acute to obtuse. So as to be sure that the intensity variation does not contain orientational effects, scattering curves were measured at fixed sample positions for various microtubule orientations

produced by suitable rotations about sample axis. The scattered intensity, after correction for geometrical factors, was independent of orientation to within 4%. Scanning a microtubule sample that did not form a self-organised structure yielded almost no variation in scattered intensity with position.

The fluorophore, 4′,6-diamidino-2-phenylindole (DAPI), is strongly fluorescent (emission 450 nm) when bound to microtubules, but only weakly fluorescent when associated with free tubulin or in buffer solution [32]. Self-organised microtubule structures were prepared containing 5 μM DAPI.

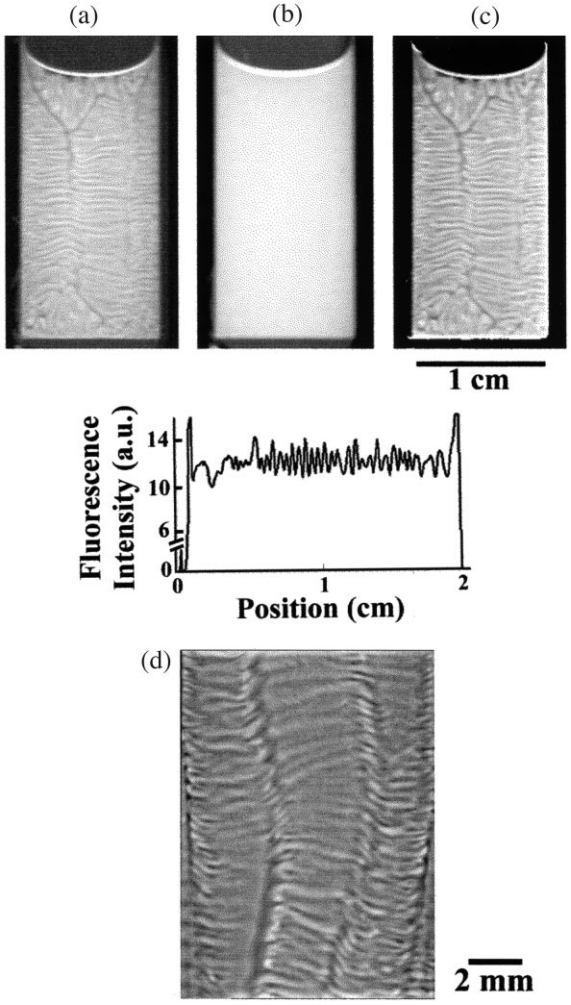

Fig. 2

The striped fluorescent pattern observed (Fig. 2a) is interpreted as arising from periodic variations in microtubule concentration. To check that this pattern does not arise from other optical properties of the solution, samples also contained another fluorophore, rhodamine chloride, whose fluorescence occurs at higher wavelength (emission 610 nm), and is independent of microtubule formation. As expected, the rhodamine image (Fig. 2b), as obtained from the same sample by changing the filters, is almost uniform. Dividing the DAPI image by the rhodamine image corrects the DAPI image, both for this effect and for variations over the sample of incident light intensity and camera response. Fig. 2c shows such a corrected image. Periodic changes of the order of 25% of the mean occur. In agreement with neutron scattering, variations in microtubule concentration coincide with changes of orientation from acute to obtuse. In addition to showing variations in microtubule concentration, these images also demonstrate that molecules that specifically bind to microtubules can show a similar concentration pattern.

The preparations show other properties expected of chemically dissipative reaction–diffu-

Fig. 2 Microtubule concentration patterns as shown by fluorescent imaging. The fluorophore DAPI (emission, 450 nm) is strongly fluorescent when associated with microtubules but only weakly fluorescent when associated with free tubulin in buffer. Rhodamine chloride is a fluorophore (emission, 610 nm) that does not bind to microtubules. Microtubule samples [4–7] were prepared containing 10 mg/ml of tubulin, 5 μM DAPI and 95 μM rhodamine chloride. The DAPI image before assembly of the microtubules was uniformly dark. Photographs (a) and (b) show DAPI and rhodamine images of the same sample after formation of the self-organised structure. The rhodamine image is close to uniform, thus confirming that the DAPI image arises from periodic variations in microtubule concentration. (c) Shows the DAPI image corrected for variations in incident light intensity and camera response by dividing it by the rhodamine image. Also shown is the DAPI fluorescent intensity along a line parallel to the long axis of the cell. In agreement with neutron scattering, the microtubule concentration variations are approximately 25%. (d) Shows the DAPI image at higher magnification. The smaller 100-μm separations can be discerned showing that this sub-patterns is also comprised of microtubule concentration variations.

sion systems. Namely, a dependence of the self-organised morphology upon weak external fields such as gravity, and a bifurcation associated with a chemical instability [4–7]. Moreover, the self-organising process contains reactive and diffusive contributions. Neutron small angle scattering shows that the stripes form by a reactive mechanism in which macroscopic domains of oriented microtubules disassemble and then reassemble in the orthogonal orientation. The periodicity of the stripes depends on the rate of reaction in a manner in quantitative agreement with reaction–diffusion theory. Increasing the viscosity of the solution by the addition of agarose gels progressively inhibits stripe formation. This and other data are consistent with a diffusive contribution to the self-organising process.

During embryogenesis, concentration patterns in specific gene products play a major role in determining the morphology of the phenotype. The early stages of *Drosophila* embryogenesis occur by way of consecutive nuclear divisions in an uncompartmentalised egg. Organisation of the cytoplasm by way of microtubules plays a role in *Drosophila* morphogenesis. Immunofluorescence observations of microtubules in the early stage of *Drosophila* eggs embryogenesis detect a striped pattern [14] similar both to those that arise in the in vitro microtubule solutions, and to segmentation gene patterns [15–17]. The question arises as to whether this pattern arises from reaction–diffusion processes, and we hence wanted to know if the pattern followed the physical–chemical laws expected for a self-organised reaction–diffusion structure.

The periodicity of a reaction–diffusion structure depends on the reactivity and diffusivity of the reacting species. In addition, due to boundary conditions in the non-linear partial differential equations describing the self-organising process, the morphology of the overall pattern also depends on the dimensions and shape of the sample container [2,3,13,26–29]. The dependence of the morphology on dimensions hence provide an experimental test for this type of system that can be used on in vivo systems such as uncompartmentalised *Drosophila* eggs.

When microtubule self-organised structures are prepared in cylindrical ‘egg’ shaped containers, the morphology shown in Fig. 3 arises. There are two stripe free regions at each end of the sample, separated by a striped central zone. Below a certain critical sample length the striped central region does not form. For longer samples, the end zones remain of the same length, and the number of stripes in the central region increases with sample length. This type of behaviour agrees with theoretical predictions for certain types of reaction–diffusion systems [8–13,26–29]. For samples of appropriate length, morphologies containing 6–7 blue and yellow birefringent stripes arise. When observed by fluorescence, this number doubles to give 12–14 microtubule concentration stripes (Fig. 3b). The pattern can be characterised both by the number of stripes and by the ratio (M) of the average length of the stripe free extremity divided by the average distance between concentration maxima. In the present case, $M = 5.0 \pm 0.5$. To check whether or not similar processes might arise in containers having the dimensions of a *Drosophila* egg, microtubules were assembled in capillaries of 150-μm radius. Striped structures having a periodicity of 100 μm formed.

After laying, *Drosophila* eggs develop by a sequence of 10 nuclear divisions in an uncompartmentalised medium [38]. Between the 10 and 14 divisions, cells progressively form at the egg surface. These cells remain open to the egg cytoplasm at the interior until gastrulation occurs, at the end of the 14th division, with the appearance of the ventral and cephalic furrows. As mentioned, the presence of striped microtubule structures in *Drosophila* fly eggs has been reported by Callaini [14]. He also showed by scanning electron microscopy that the egg surface was as flat and without periodic bulges. In addition, the distribution of actin in the egg at the moment that the microtubule stripes are detected, is uniform. Using the same fluorescence procedure, we obtained the striped microtubule pattern shown in Fig. 4a. Although the contrast is low, 12 stripes can be definitely counted, yielding $M = 5.3 \pm 0.3$, and there may be two additional stripes of lower intensity. The structure appears briefly for approximately 5 min at gastrulation. This is the last stage

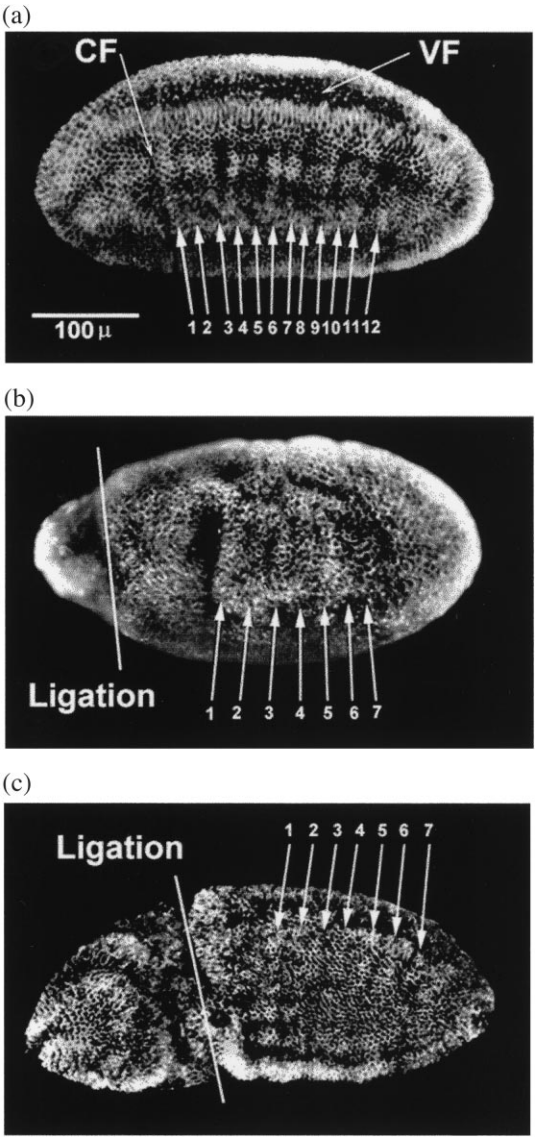

Fig. 4

at which the non-compartmentalised internal cytoplasm is still connected by the cytoplasm of the epithelial layer cells [33].

As shown above, a characteristic feature of the in vitro patterns is their dependence upon sample dimensions. *Drosophila* eggs can be shortened by ligating them shortly after laying [34–37]. Within 10 min a membrane forms that separates the egg into two unconnected fragments. Development can occur in either one or sometimes both parts of the egg. We examined the microtubule pattern formed in *Drosophila* eggs ligated[2] at the 9th–10th nuclear division, 2 h before gastrulation, as a function of fragment length (Fig. 4b–c). Although the contrast is low, and it is difficult to count the exact number of stripes, nevertheless the pattern is clearly comprised of two stripe-free extremities separated by a central striped region. The length of the end zones is independent of fragment length and approximately the same as in unligated eggs. Moreover, in the ligated egg shown in Fig. 4c, both fragments have continued to develop. However, there are no stripes in the shorter fragment. This is consistent with the fact that in this case the fragment length is below the critical length necessary for stripe formation.

This behaviour resembles that of the in vitro

Fig. 4 Microtubule patterns in whole and ligated *Drosophila* eggs. Microtubules observed by immunofluorescence, in (a) whole, and (b,c) ligated *Drosophila* eggs. Eggs at 22°C were ligated at the 9–10th nuclear division, 2 h 10 min after laying, and fixed at gastrulation, 4 h after laying, using the procedure described by Callaini. The position of the ventral and cephalic furrows, indicating gastrulation, are shown in (a). Ligation divides the egg into two unconnected fragments, and development continues in one, or occasionally both, of the fragments. The survival rate for ligated eggs reaching gastrulation was approximately 10%. There was a further 50% loss during fixation and staining. All the embryos were followed individually so as to fix and stain the eggs at the appropriate developmental stage. In the complete egg, the microtubule pattern is comprised of a striped central region and stripe free ends. For fragments below a minimum length, stripes are not observed. This is seen in the anterior fragment of the egg shown in (c), in which both fragments have developed. For longer fragments, the pattern is comprised of stripe free extremities separated by a striped central region. Although it is not easy to count the number of stripes, the length of the end zones is independent of fragment length, and similar to that of unligated eggs. The length of the striped central region decreases with decreasing fragment length. The overall behaviour resembles that of in vitro microtubule samples and is evidence that similar reaction–diffusion processes occur in both cases.

[2] Eggs of the white *Drosphila melanogaster* strain (gift from R. Griffin) were collected and ligated as described [34–37]. Fixation and immunostaining followed published procedure [14,39] except that the second antibody (gift from D. Job) contained a cyanine-3 group as fluorphore. Eggs were observed on a Zeiss Axioplan microscope using a Neofluar 25 × /0.8 objective, and photographed with Kodak Technical Pan film.

samples and is evidence that similar reaction–diffusion processes occur in both cases.

The results presented here provide experimental evidence that reaction–diffusion processes spontaneously generate microtubule concentration patterns during *Drosophila* embryogenesis. In spite of the fact that developing eggs contain numerous molecular components, the microtubule patterns that arise both in vitro and in vivo, nevertheless behave in a manner similar to one another and consistent with reaction–diffusion theory. The formation of these concentration patterns by way of such processes may provide a way by which a predetermined macroscopic pattern spontaneously arises in an initially unstructured egg.

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