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# Embryonic nodal flow and the dynamics of nodal vesicular parcels

Julyan H. E. Cartwright<sup>1</sup>, Nicolas Piro<sup>2</sup>, Oreste Piro<sup>3,4</sup> and Idan Tuval<sup>5,\*</sup>

<sup>1</sup>*Laboratorio de Estudios Cristalográficos, CSIC, 18100 Armilla, Granada, Spain*

<sup>2</sup>*ICFO-Institut de Ciències Fotòniques, Mediterranean Technology Park, 08860 Castelldefels (Barcelona), Spain*

<sup>3</sup>*Institut Mediterrani d'Estudis Avançats, CSIC-UIB, 07071 Palma de Mallorca, Spain*

<sup>4</sup>*Center for Studies in Physics and Biology, Rockefeller University, New York, NY 10021, USA*

<sup>5</sup>*Bio5 Institute, University of Arizona, Tucson, AZ 85721, USA*

We address with fluid-dynamical simulations using direct numerical techniques three important and fundamental questions with respect to fluid flow within the mouse node and left–right development. First, we consider the differences between what is experimentally observed when assessing cilium-induced fluid flow in the mouse node *in vitro* and what is to be expected *in vivo*. The distinction is that *in vivo*, the leftward fluid flow across the mouse node takes place within a closed system and is consequently confined, while this is no longer the case on removing the covering membrane and immersing the embryo in a fluid-filled volume to perform *in vitro* experiments. Although there is a central leftward flow in both instances, we elucidate some important distinctions about the closed *in vivo* situation. Second, we model the movement of the newly discovered nodal vesicular parcels (NVPs) across the node and demonstrate that the flow should indeed cause them to accumulate on the left side of the node, as required for symmetry breaking. Third, we discuss the rupture of NVPs. Based on the biophysical properties of these vesicles, we argue that the morphogens they contain are likely not delivered to the surrounding cells by their mechanical rupture either by the cilia or the flow, and rupture must instead be induced by an as yet undiscovered biochemical mechanism.

**Keywords:** left–right development; biophysical fluid dynamics; nodal flow; asymmetry

## 1. INTRODUCTION

The determination of left and right in the body plan, in the mouse at least, and possibly in other vertebrate embryos (Essner *et al.* 2002), appears to originate with a fluid flow (Nonaka *et al.* 1998). The extraembryonic fluid filling the node, a closed depression on the ventral surface of the embryo (figure 1), is set into motion by motile cilia that bend like ropes being whirled in circles. *In vitro* observations with bead-tracking experiments show a strong leftward current across the node in wild-type mouse embryos that develop normal situs (Nonaka *et al.* 1998), while an artificial reversal of this current leads to an embryo developing situs inversus (Nonaka *et al.* 2002). Recently, we demonstrated with fluid-dynamical simulations (Cartwright *et al.* 2004) that tilted cilia would produce the required directional flow. In an infinite system—in the absence of walls—there is a flow above the cilia in one direction and another in the opposite direction below them. Upon putting such a set of cilia inclined towards the posterior into a closed system representing the *in vivo* node, there is a leftward

flow across the middle of the node together with rightward recirculation of fluid around the walls. From this, we anticipated that the nodal cilia should be tilted towards the posterior. This prediction has now been confirmed by new experimental observations of the node (Okada *et al.* 2005; Nonaka *et al.* 2005). However, important points remain to be understood. In the experimental work, Reichert's membrane covering the node is of necessity removed. To what degree does the observable *in vitro* flow with the membrane removed differ from the unobservable *in vivo* flow with it in place? Is it for this reason that the return flow predicted in our earlier work on the *in vivo* case has not been observed experimentally in the *in vitro* flow?

Furthermore, the latest experimental observations of the node (Tanaka *et al.* 2005) show that the mechanism by which the symmetry breaking, provided by the flow direction, is passed on to the rest of the embryo is rather different from the hypotheses postulated previously. Some had argued for mechanical sensing of the flow; while in our earlier work we had indicated the physical difficulties with mechanical sensing and had modelled chemical sensing with a diffusible morphogen in the nodal flow. But rather than

\*Author for correspondence (idan@email.arizona.edu).

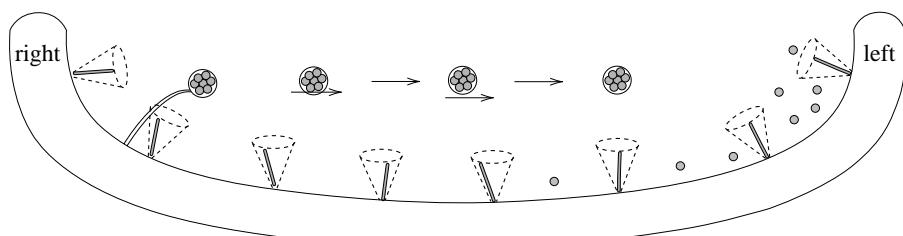


Figure 1. Sketch of a vertical slice across the node viewed from the ventral side showing the monocilia producing the leftward flow that transports NVPs. The mouse node is some 50  $\mu\text{m}$  across by 10  $\mu\text{m}$  depth. Note that, following the convention in this field, in this and all subsequent vertical slices of the node shown here, the node is seen from the ventral side, and thus the left side of the embryo is on the viewer's right.

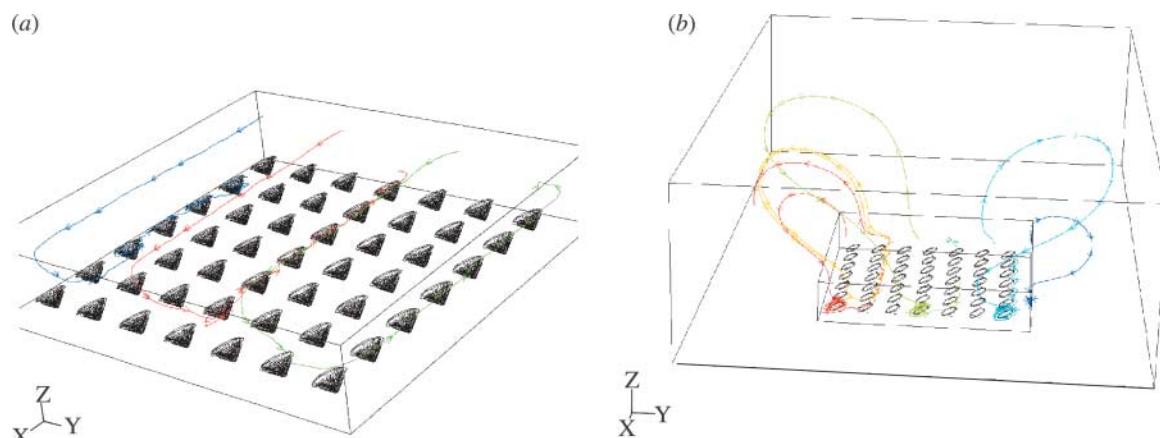


Figure 2. General three-dimensional views of particle trajectories in simulated nodal flow: (a) the upper recirculation within the *in vivo* node and (b) the equivalent situation *in vitro*.

this type of direct interaction of a morphogen with the extraembryonic fluid in the node, new observations have shown that the nodal flow in fact transports small particles, termed nodal vesicular parcels (NVPs), across the node (Tanaka *et al.* 2005). These are natural passive tracers, some 0.3–5  $\mu\text{m}$  in diameter, that contain morphogens—sonic hedgehog and retinoic acid—within a lipid membrane. When launched, these pass intact through the fluid flow, but apparently break upon impact with the walls of the node, to deliver the morphogens they contain. Here, we take these new observations into account in direct numerical simulations of the hydrodynamics of the node; furthermore, we demonstrate that there must be a hitherto undiscovered biochemical mechanism for the rupture of these NVPs.

## 2. METHODS

We represent the mouse node by a fluid-filled box of dimensions  $50 \times 50 \times 10 \mu\text{m}$ , either completely closed as *in vivo* or open at the top and placed within a much larger fluid-filled volume to model the *in vitro* bead-tracking experiments, which take place in such an experimental set-up. We solve the steady-state Navier–Stokes equations for the nodal fluid set into motion by the cones that form the surfaces of revolution of the cilia. These (length 3  $\mu\text{m}$ ; half-angle 45°; rotation frequency 10 Hz) rotate clockwise when viewed from above, inclined at an angle  $\alpha = 25^\circ$  to the posterior. The ciliary Reynolds number is of the order of  $10^{-4}$ .

The nodal flow is thus in the so-called creeping-flow regime. We used this fact in our previous work (Cartwright *et al.* 2004) to model analytically each cilium by an exact solution of the Stokes equation. However, in the direct numerical simulations presented here, we have in fact solved the full Navier–Stokes equations. We solve the equations numerically using a finite-element method on a tetrahedral mesh. We use a mesh of 100 by 100 points in the  $x$  and  $y$  directions, and 40–50 points in the  $z$  direction. The cilia are modelled as the cones that are their surfaces of revolution—the surface generated by revolving a cilium about its rotation axis. The spatial mesh has 10–20 points in the vertical direction on each cilium, and a similar number about its circumference. We minimized numerical errors by optimizing the computational mesh, numerical scheme and convergence criteria. Our results did not change on increasing the fineness of the mesh.

In our simulations, we have implicitly idealized the node in various ways. In the first place, we have considered the node as a box. Of course, the node does not have sharp edges and corners, but in fluid-dynamical terms this difference is not significant. The pertinent fluid-dynamical variable, the Reynolds number in the node—the ratio of inertial to viscous forces—has been set to be the same in the simulations as in the real mouse node, and we can be confident that the fluid flow is likewise the same.

We have idealized the NVPs as passive tracers that faithfully follow the flow. This means that we can neglect any influence of the NVPs upon the nodal

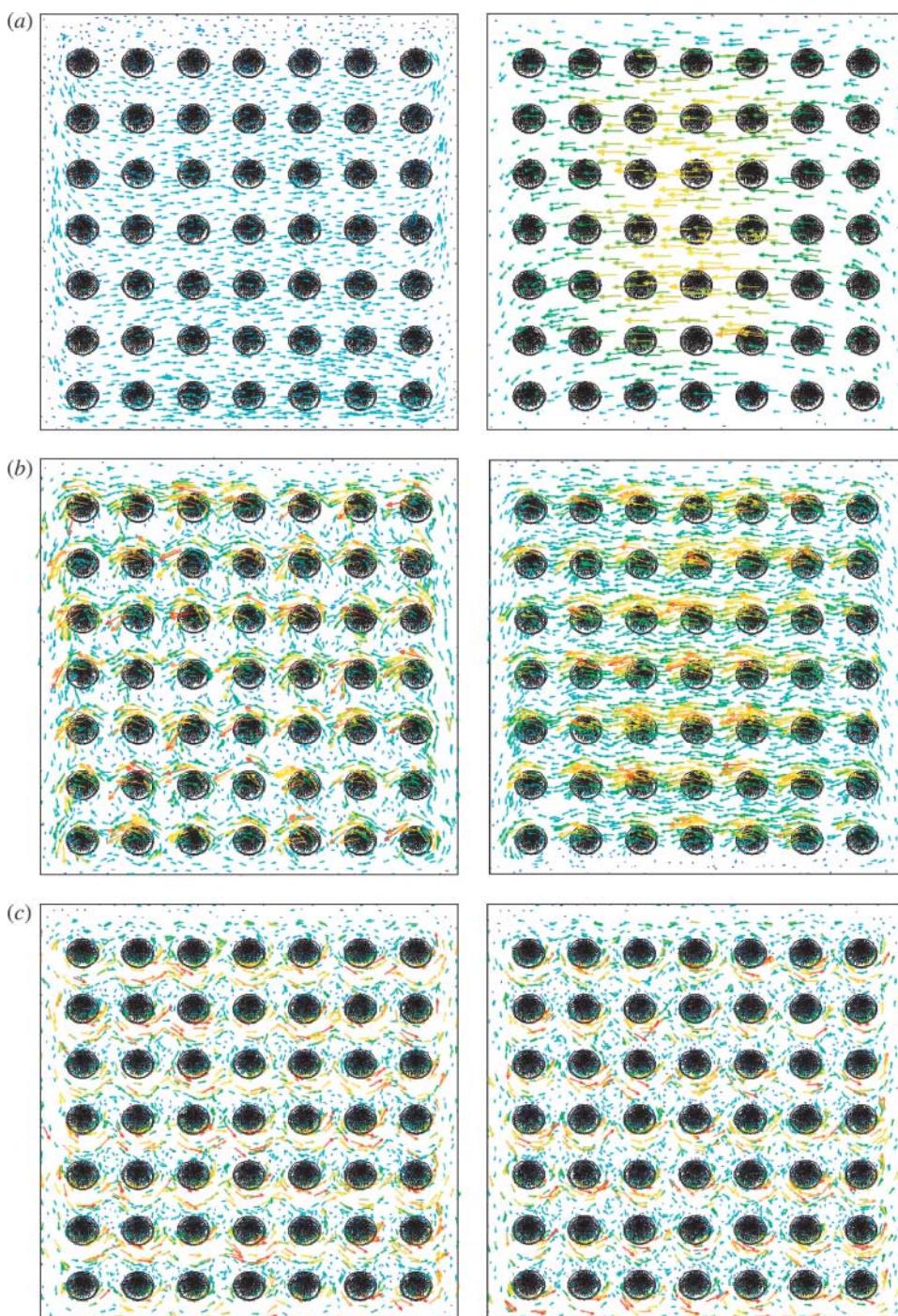


Figure 3. Horizontal slices of the velocity field in the node: on the left *in vivo* and on the right *in vitro*. (a) The upper slices are at 8  $\mu\text{m}$ , near the top of the node; (b) the middle slices are at 5  $\mu\text{m}$ , in the centre of the node; and (c) the lower slices are at 0.2  $\mu\text{m}$ , next to the floor of the node. The cilia appear in the upper slices just as a guide for the eye.

flow, as well as any finite-size effect on the NVP dynamics, and we assume them to move as determined solely by the equation of motion of a fluid parcel. This approach is valid as long as the Stokes number of an NVP—the relaxation time of the particle back onto the fluid trajectories compared to the time-scale of the flow—is small enough and the NVP density is close enough to the fluid medium density (Babiano *et al.* 2000). One unknown is thus the density of the NVPs; in the absence of further data, we suppose them to be neutrally buoyant. The NVPs will most probably be close to neutrally buoyant. We can turn the question around by noting

that otherwise, if they were too light or too heavy, they would be propelled by centripetal or centrifugal forces to the middle or to the edges of the vortices in the node. We do not observe this behaviour, and it would make them of little use for their job of transporting materials across the node. Small neutrally buoyant particles will faithfully follow a fluid flow as long as certain conditions on the flow apply: it should not deform fluid parcels to a greater extent; contrariwise, particles will cease to follow pathlines of the flow (Babiano *et al.* 2000). These conditions on what, in dynamical terms, is the hyperbolicity of the flow probably break down precisely where the NVPs rupture.

### 3. RESULTS AND DISCUSSION

#### 3.1. Nodal flow *in vivo* and *in vitro*

We present in figure 2 an overview of the circulation in the *in vivo* and *in vitro* nodal flows. In both the *in vivo* and *in vitro* node, there is a general leftward flow across the centre of the node that corresponds well to that observed in experiments (figures 3b and 4). In the *in vivo* case, this fluid recirculates within the node following the walls (figures 2a and 4a). So the general scheme is of two vortices, one in each of the upper and the lower halves of the node; fluid flows to the left across the middle and returns to the right along the ceiling (figure 3a) and floor of the node (figure 3c). The flow in the lower vortex is more complex than in the upper vortex owing to the presence of the cilia. While there is a general recirculation across the floor of the node, some pathlines representing fluid parcels become trapped in the vicinity of a cilium and may spend some time there before rejoining the general circulation. In contrast, in the *in vitro* case, the opening of the node with the removal of its covering membrane completely eliminates the upper recirculatory vortex within the node (figures 3a and 4b). The fluid is now free to enter and leave the node to flow around a much larger surrounding volume, so that the upper recirculation occurs around the whole of the box containing the node (figure 2b). As the fluid volume is larger, velocities are lower, and the rightward return current occurs far beyond the limits of the node in a manner so diffuse that it is almost imperceptible compared with the leftward flow within the node (figure 4c). The strong leftward current above the cilia is, as before, the most prominent feature of the flow (figures 3b and 4b). The flow in the lower half of the node, below the leftward current, persists, but the general rightward component is diminished compared with the recirculatory flow around the individual cilia (figure 3c). Thus, while the main feature of the nodal flow, the central leftward current, is present in both *in vivo* and *in vitro* conditions, there are significant differences in other aspects of the two flows. This may explain why the recirculation of fluid rightwards predicted in the *in vivo* case has not been observed *in vitro*, and should make one wary of using *in vitro* bead-tracking experiments as the sole basis for understanding the *in vivo* flow; they ought to be interpreted together with a knowledge of the differences between the *in vivo* and the *in vitro* flows.

While the mouse is the animal in which the fluid-dynamical aspects of left-right symmetry breaking have been most studied, similar ciliated structures are present in many other vertebrate embryos (Essner *et al.* 2002). At least in the case of the zebrafish, the fluid flow in the equivalent structure to the node, Kupffer's vesicle, is also caused by the same circular movements of the cilia (Essner *et al.* 2005; Kramer-Zucker *et al.* 2005). As in the mouse, the cilia are also seen to be tilted (Kramer-Zucker *et al.* 2005), and so we should expect a similar general circulation. Fluid flow in Kupffer's vesicle should be similar to the closed system represented by the *in vivo* case in the mouse node; the experiments demonstrating flow were carried out by the injection of beads

into Kupffer's vesicle without removing its covering membrane (Kramer-Zucker *et al.* 2005).

#### 3.2. Nodal vesicular parcels as natural passive scalars

Having understood the fluid dynamics of the *in vivo* and *in vitro* flows, we now add the NVPs into our model, with the aim of understanding their transportation by the flow. We compute the trajectories of the NVPs, assuming them to be perfect passive tracers released at random points above the floor of the node. This simulates their experimentally observed origin as vesicles are projected into the flow by microvilli and released every 5–15 s (Tanaka *et al.* 2005). In our model, again following the experimental observations, we assume their breakage when they collide with a wall or with a cilium. We can then collect the statistics corresponding to some hours of nodal flow of the position within the node at the moment of rupture of a large number of NVPs; we present the results in the histograms of figure 5. The outcome is similar for the *in vivo* and the *in vitro* cases. Most of the NVPs are transported leftwards across the node and collide with the left wall or the cilia nearest to it. The smaller intermediate peaks in the histograms indicate that few are broken in other locations across the floor of the node by interactions with the cilia there. The *in vitro* histogram shows a somewhat smaller main peak on the left side of the node than the *in vivo* case, although it is clear that in both instances the majority of NVPs break on the left side of the node. This means that no matter where the NVPs are released by the microvilli and, in particular, if they are released in a symmetric fashion across the node, they will most probably break near the left wall and deliver there their cargo of morphogens.

The results shown in figure 5 depend on the gross features of the flow—primarily the existence of the leftward flow across the tops of the cilia—and so as figure 5 itself demonstrates, even on completely changing the geometry by opening the flow, the results are not vastly different because this leftward flow is preserved. The other pertinent variable is the position of release of the NVPs; they need to find their way into this leftward flow. Again, figure 5 itself demonstrates that the results are robust with regard to changes in the exact position of release, since it shows statistics collected by releasing NVPs at different points within the flow. The majority of NVPs do get caught up in the leftward flow, while a few are entrained into the vortices around each cilium and collide at other points along the nodal floor.

#### 3.3. Rupture of nodal vesicular parcels

A membrane will rupture if it is forced to stretch—to increase its surface area—beyond a critical threshold. Rupture generally occurs in biological membranes for a critical applied stress, the lysis tension, at which point the membrane surface area has been increased by some 2–5% (Boal 2002). A useful analogy to visualize how this applies here is to imagine the NVPs containing their cargo of morphogens as sacks of potatoes. The sack—the membrane—is easily deformable without an

increase in the surface area if the sack is loosely filled, but on the other hand, if it is completely filled, any attempt at deforming it leads to an increase in the surface area, with the consequent possibility of breaking the sack, i.e. rupturing the membrane. We should then consider two alternatives for the NVPs: either the membrane is taut, so that the vesicle is maintained approximately spherical, or it is slack, so that the vesicle can deform from the state of sphericity without breaking.

Images of NVPs attached to microvilli in the node show these to be projecting out from the nodal surface into the flow with an appending NVP (Tanaka *et al.* 2005). This presumably adheres to its microvillus by electrostatic, van der Waals or hydration forces. The microvillus could either actively release its NVP by decreasing the adhesive forces, or else detachment could occur with no input on the part of the microvillus, if the NVP breaks away once the microvillus projects far enough out into the flow so that the Stokes drag given by the local flow velocity exceeds the adhesive forces. It then circulates in the flow until it ruptures, apparently upon impact with a cilium or with the node wall, releasing its cargo of morphogens (sonic hedgehog and retinoic acid (Tanaka *et al.* 2005)) held within the membrane. The average Stokes drag force suffered by the NVPs at the point of being released into the flow, taking them to be spherical, is given by  $F_D = 6\pi\mu Ur$ , where  $\mu$  is the viscosity of the fluid,  $U$  is the magnitude of the nodal flow velocity and  $r$  is the radius of the NVP. To obtain a conservative estimate, let us assume that the viscosity of the extraembryonic fluid is not dissimilar to that of water,  $\mu \approx 1 \text{ mPa s}$ , the average vesicle radius is  $r \approx 1 \mu\text{m}$ , and the average node flow velocity is  $U \approx 4 \mu\text{m s}^{-1}$ . This gives an average Stokes drag of  $F_D \approx 8 \times 10^{-14} \text{ N}$ ; this is a lower bound, as some of the above terms could be an order of magnitude larger. Since the NVP is attached to the microvillus via its covering membrane,  $F_D$  corresponds to the force on the membrane at the moment of detachment. This is the force if the vesicle remains spherical, while if the membrane is slack, the vesicle will deform itself to minimize drag (Abkarian & Viallat 2005) and this force can be in an order of magnitude less. What about the viscous forces on a vesicle within the flow; can they rupture the vesicle? These, in the most extreme case, can be greater than the Stokes drag; a sphere at the centre of an X-shaped flow, where fluid approaches from two opposite directions, squeezing the sphere, and recedes in the other two, stretching it, experiences a force tending to distort it from the sphericity of  $F_V = 32\pi\mu Vr$ , where  $V$  is the flow velocity at the position of the surface of the sphere if the sphere were not present (Taylor 1932). However, in the present instance this force is unlikely to exceed the Stokes drag at the moment of detachment because away from the wall, where flow velocities are large, the shear is very much lesser than this, while close to the wall, where shear flow, albeit not the extreme type detailed above, occurs,  $V \ll U$ .

It has been suggested that NVPs rupture on impact with cilia or with the node wall; certainly, they appear to break in the vicinity of cilia or the wall, but is it really

an impact process? This would be remarkable, as the Reynolds number of a NVP moving in the flow is rather low; using the numbers given above,  $Re = \rho Ur/\mu \sim 4 \times 10^{-6}$ , viscous forces dominate over inertia. This would imply that any impact force should be much less than the Stokes drag, and so it should be incapable of rupturing the membrane. Let us estimate the impact force and compare it with the Stokes drag on an NVP as it is released into the flow to illustrate our point. The impact force suffered by the NVPs when they collide with a cilium or a wall can be estimated by Newton's second law. If  $\Delta V$  is the relative velocity of the NVP with respect to the node wall or a cilium, and  $\Delta t$  is the impact time, then the average force suffered by an NVP during a collision is  $F = m\Delta V/\Delta t = m\Delta V^2/(2l_d)$ , where  $l_d$  is the deformation length suffered by the NVP due to the impact and  $m$  is the mass of the vesicle. This mass is given by  $m = 4/3\pi r^3 \rho$ , where  $\rho$  is the density of the NVP, which we shall assume to be similar to that of the extraembryonic fluid (there is an additional factor of the added mass of moving fluid surrounding the NVP, which we can effectively build into this by taking  $r$  to be the radius of the vesicle and the surrounding comoving fluid). If  $\beta = l_d/r$  is the fraction of its radius that the NVP deforms during the collision, then the impact force is given by  $F_I = 2/3\pi r^2 \rho \Delta V^2 / \beta$ .  $\beta$  is a measure of the packing within the NVP; a loosely filled parcel can deform a great deal, while a tightly packed parcel can deform only a small amount. If we assume that the density of the extraembryonic fluid is approximately that of water,  $\rho \approx 10^3 \text{ kg m}^{-3}$ , and consider that in the most energetic impact possible, a head-on collision with a cilium moving in the opposite direction,  $\Delta V \approx 100 \mu\text{m s}^{-1}$ , we obtain  $F_I \approx 2 \times 10^{-19} / \beta \text{ N}$ . This impact force is an upper bound on the force experienced by the membrane on impact: all the impact force would be transmitted to the membrane only if there were no internal dissipation of the impact energy, and consideration of the sack-of-potatoes model indicates that it is likely that much of the energy would be dissipated internally. Thus, we have a conservative estimate of the force on the membrane at the moment of vesicle release of  $F_D \approx 8 \times 10^{-14} \text{ N}$ , while on collision the force on the membrane is at most  $F_I \approx 2 \times 10^{-19} / \beta \text{ N}$ . Since the vesicle ruptures during impact, and not during release, we suppose  $F_I > F_D$ , which would imply  $\beta < 2.5 \times 10^{-6}$ , that is to indicate that the vesicle must be very tightly filled, so that it can only deform by at most a few parts per million before stretching its covering membrane to breaking point. If the membrane were this taut, it would certainly have ruptured previously; either the Stokes drag force experienced on release or the deformation caused by shear within the viscous flow, would certainly rupture such a fragile structure within the flow long before it could deliver its cargo.

The inexorable conclusion of physics is that, rather than just the mechanical breakage of the vesicles, there must exist an active rupture mechanism that acts upon the impact of the vesicle with a particular region of the node or the cilia. In other words, the vesicles are not broken by mechanical forces, so we must entertain the idea that contact of the vesicular membrane with something on the wall of the node or with certain cilia

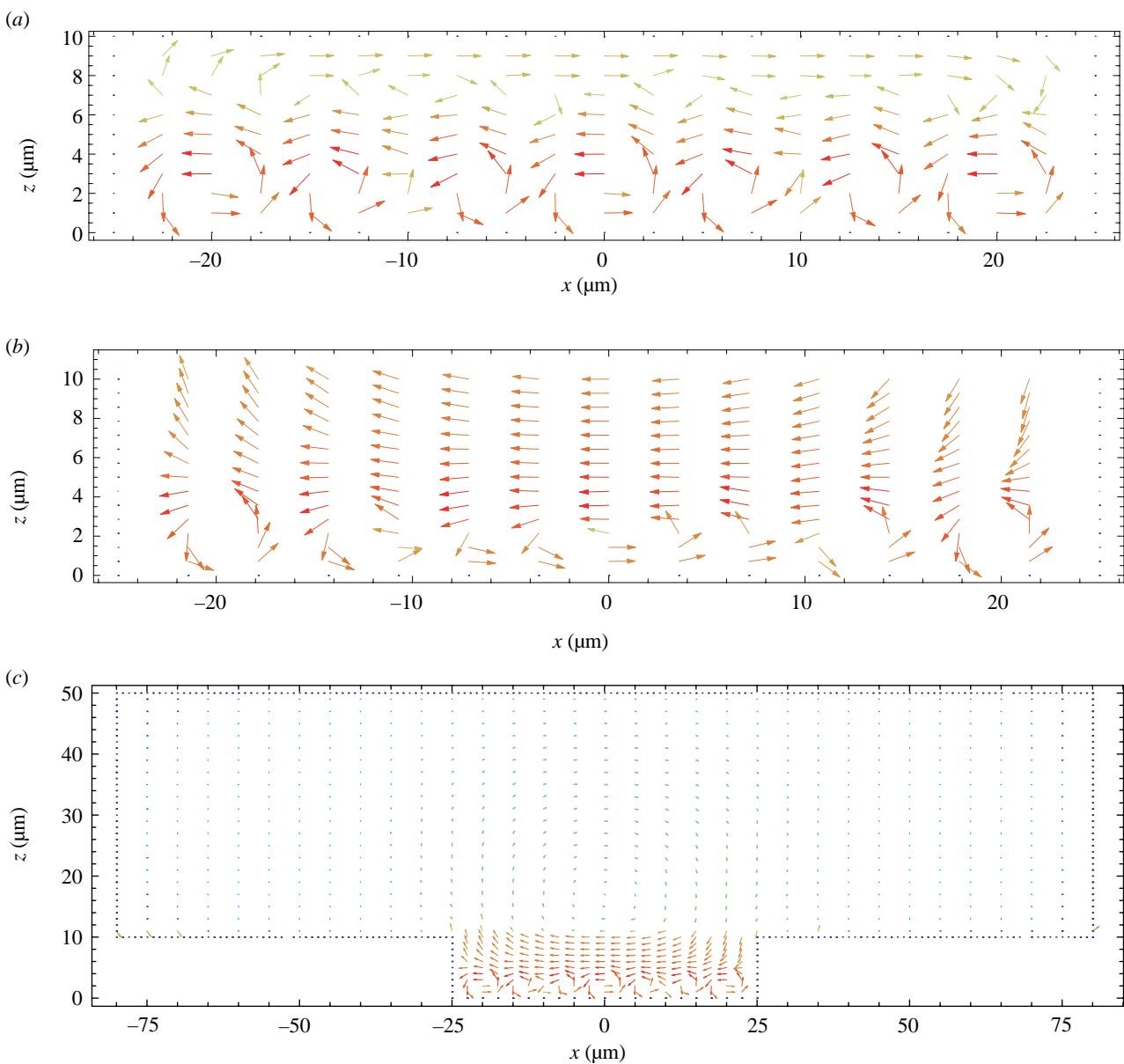


Figure 4. Vertical views corresponding to  $y$ -direction averages of the velocity field in the node: (a) *in vivo* and (b) *in vitro*. (c) General view of the *in vitro* node immersed within a container of much larger volume; so (b) is seen at bottom centre.

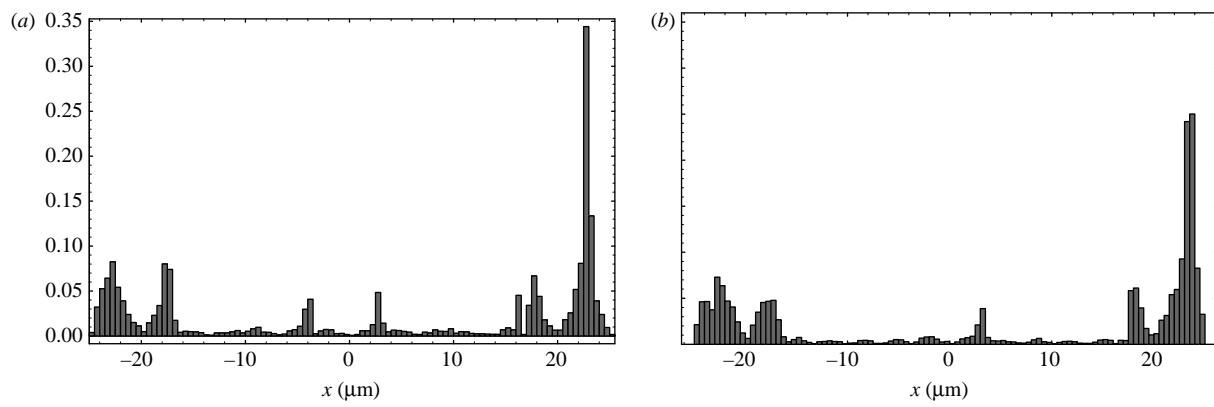


Figure 5. Histograms showing the relative frequency with which a NVP breaks as a function of its position from right to left along the floor of the node. (a) The *in vivo* case and (b) the *in vitro* flow.

destabilizes the membrane by means which might be presumably chemical in nature. Mutant mouse embryos in which NVPs are released, but cilia are immobile, offer a datum in favour of our argument that

there is an undiscovered biochemical rupture process for the NVPs. In these embryos, rupture of NVPs appears to occur even without the flow (Tanaka *et al.* 2005). If the flow mechanism is faulty in this mutant,

but the—separate—biochemical contact rupture mechanism is functioning, these results are explained. Might the active regions involved in this rupture mechanism be associated with the second population of apparently immotile cilia recently found towards the sides of the node (McGrath *et al.* 2003)? This idea would serendipitously unite the rival morphogen and two-cilia models on how nodal flow is interpreted by the embryo: these cilia could be mechanosensors as originally postulated in the two-cilia model, while concurrently acting to break up the NVPs via a biochemical mechanism to release the morphogens within, as hypothesized in the earlier morphogen model. But the chemical signal would not have to be localized to the periphery of the node for the NVPs to dissociate primarily in this location; this remains a function of the frequency with which they arrive in proximity with the wall at different locations across the node, as we showed earlier. We therefore conclude by postulating an active biochemical mechanism for the fragmentation of NVPs.

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## NOTICE OF CORRECTION

On page 2, in the methods section, the dimensions of the fluid-filled box are now correctly given as  $50 \times 50 \times 10 \mu\text{m}$ .

On page 6, the cilium impact velocity is now correctly given as  $\Delta V \approx 100 \mu\text{m s}^{-1}$ .

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