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French flag gradients and Turing reaction-diffusion versus differentiation waves as models of morphogenesis

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ABSTRACT

The Turing reaction-diffusion model and the French Flag Model are widely accepted in the field of development as the best models for explaining embryogenesis. Virtually all current attempts to understand cell differentiation in embryos begin and end with the assumption that some combination of these two models works. The result may become a bias in embryogenesis in assuming the problem has been solved by these two-chemical substance-based models. Neither model is applied consistently. We review the differences between the French Flag, Turing reaction-diffusion model, and a mechanochemical model called the differentiation wave/cell state splitter model. The cytoskeletal cell state splitter and the embryonic differentiation waves was first proposed in 1987 as a combined physics and chemistry model for cell differentiation in embryos, based on empirical observations on urodele amphibian embryos. We hope that the development of theory can be advanced and observations relevant to distinguishing the embryonic differentiation wave model from the French Flag model and reaction-diffusion equations will be taken up by experimentalists. Experimentalists rely on mathematical biologists for theory, and therefore depend on them for what parameters they choose to measure and ignore. Therefore, mathematical biologists need to fully understand the distinctions between these three models.

1. Introduction

Models allow us to consider and explore how a phenomenon occurs. The Turing reaction-diffusion model and the French Flag Model are widely accepted in the field of development as the best models for explaining embryogenesis. Since 1952, the model of choice has been Alan Turing's reaction-diffusion driven instability (Turing, 1952). Lewis Wolpert introduced the French Flag model in 1968 (Wolpert, 1968) and elaborated it into the concept of positional information in 1969 (Wolpert, 1969). Virtually all attempts to understand cell differentiation in embryos begin and end with some combination of these two models. However, the result may become a bias in embryogenesis by assuming that the problem has been solved by these chemical substance-based models even when the models have clearly failed (Chhabra et al., 2019).

In addition to the Turing reaction-diffusion model and the French Flag Model, there have also been physico-chemical models (or mechanochemical models). The problem of tissue folding (morphogenesis without considering causes of cell differentiation) had been analyzed as a problem in laminate mechanics since at least Wilhelm His in 1874 (Gordon, 1999; His, 1874, 1888). These alternative models have been proposed and developed for embryogenesis (Brodland, 2011; Fletcher et al., 2017; Gordon and Brodland, 1987; Nikolopoulou et al., 2017) but largely ignored by biologists. Why is this so? In 1924, the biological science community saw a double embryo resulted experimentally (Spemann and Mangold, 1924, 2001) (Fig. 1). The dorsal lip of the blastopore was called the "organization center" or "organiser" because it seemed to have the ability to organize, or induce an entire new secondary embryo from the surrounding tissue of the host. Many substances were then tested for the ability to induce. Biologists tried substances as diverse as fish liver (positive result) and banana peels (no effect), reviewed in (Gordon, 1999). The astonishing variety of substances that could act as inducers clearly implicated physico-chemical causes. However, any analysis of physico-chemical model would have required nonlinear multiphysics finite element and computing capabilities which

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simply did not exist at that time.

Such research has just begun (Brodland, 2011, 2015; Brodland et al., 2010; Crawford-Young et al., 2018; Fletcher et al., 2017; Gleghorn et al., 2013; Lu et al., 2015; Nikolopoulou et al., 2017). Partially because of the bias against physico-chemcial models, virtually all model of embryo cell differentiation assume diffusing molecules acting as morphogens. No 'physico-chemical' model was proposed until Gordon and Brodland in 1987 (Gordon and Brodland, 1987). Their differentiation wave/cell state splitter model is considered as a mechanochemical model and represents a radical departure back to what was considered the insoluble approach. The result is that very few scientists are even aware that a mechanochemical model of development exists even as their results make them demand a new model (Chhabra et al., 2019).

It used to take supercomputers to work out the consequences of models of morphogenesis (Hunding, 1991, 1993; Hunding et al., 1990). However, with the increase in speed of computers by factors of 10^4 to 10^8 since 1990, following Moore's Law (Colleaga, 2019; Moore, 1965; Sneed, 2015), computational morphogenesis proceeds apace (Igamberdiev et al., 2018).

In this mini-review paper, we explain the fundamental differences between the French flag gradient model, the Turing reaction-diffusion model, and the differentiation wave/cell state splitter model (Gordon and Gordon, 2016a, b; Gordon, 1999) so that the distinctions between these three models can be understood. It has been suggested that these concepts may not be clear to the mathematical biology community. For instance, Fig. 1E in Chen and Zou (2019), showing a morphogen gradient in the context of the French flag model of embryogenesis, was incorrectly cited as being from the book *Embryogenesis Explained* by Gordon and Gordon (2016a) who do not accept the idea that gradients direct embryogenesis. Rather Gordon and Gordon regard gradients as an epiphenomenon produced in the wake of mechanochemical differentiation waves. We hope that this paper can make mathematical biologists pay more attention to the existence and development of 'physico--chemical' models in the study of embryogenesis. Eventually, the development of theory can be advanced. Moreover, we hope that experimentalists will take up those observations relevant to distinguishing the embryonic differentiation wave model from the French Flag model and reaction-diffusion equations.

2. The Turing reaction-diffusion model

The history of the idea of gradients in morphogenesis was reviewed by Charles Child (1941), and goes back at least to Theodor Boveri in 1901 (Boveri, 1901). A gradient was regarded as a monotonic function along a single direction, such as $C(x)$. In 1952 Alan Turing coined the word morphogen for molecules in spatiotemporally oscillating (sometimes) concentration gradients generated by at least two interacting kinds of molecules with different diffusion coefficients (Turing, 1952) (Fig. 2). A Turing gradient is a vector field $T_m(\boldsymbol{r}_n, t)$ whose m components are concentrations of $m \geq 2$ chemically interacting substances in a Euclidean space $\boldsymbol{r}$ of n dimensions, and t is time. In the Turing reaction-diffusion (RD) model, because some solutions are spatially periodic, positional information cannot be defined uniquely, i.e., there may be no one-to-one mapping between concentration of a given substance and coordinate along a given direction. Nevertheless, Turing did assume that a cell changes kind by reading and responding to the local concentration of at least one of the two or more morphogens, resulting in a periodic pattern of cell differentiation.

Turing's RD model has received increasing attention for tissue pattern formation and has been extended by Gierer, Meinhardt and

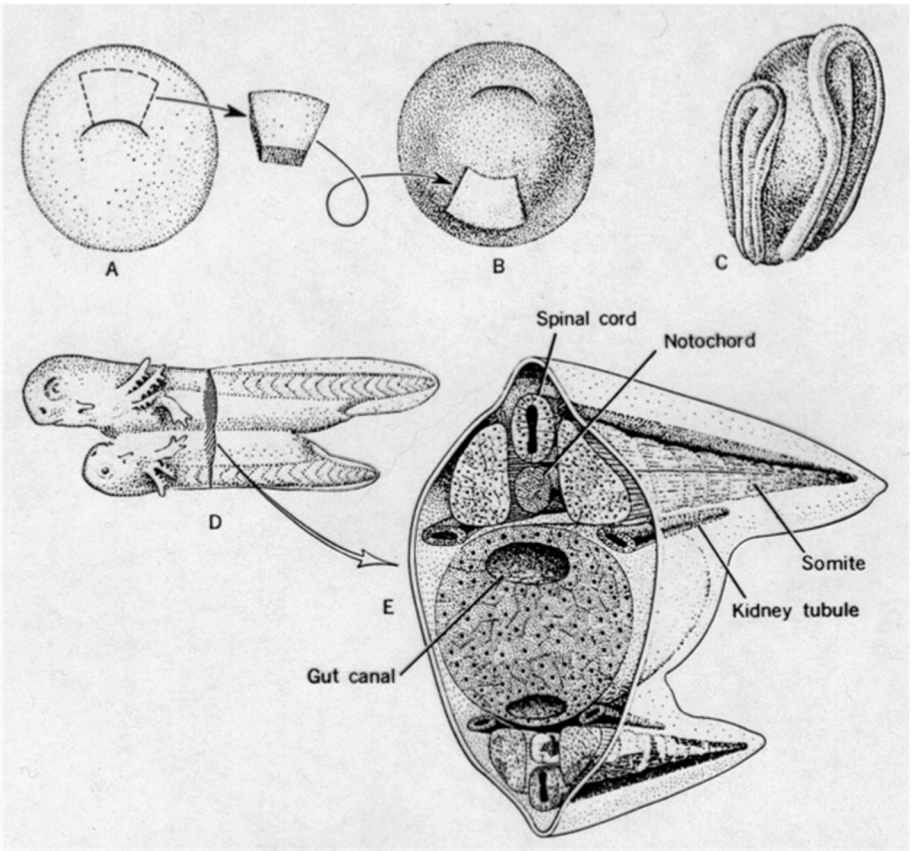

Fig. 1. Transplantation of the dorsal lip of the blastopore from (A) the embryo of one light colored species of salamander to (B) an embryo of a darkly pigmented species resulted in two neural plates consisting of cells from the host, which further developed into conjoined twins, from (Twitty, 1966) (Gordon and Gordon, 2016b) with permission of Macmillan Education.

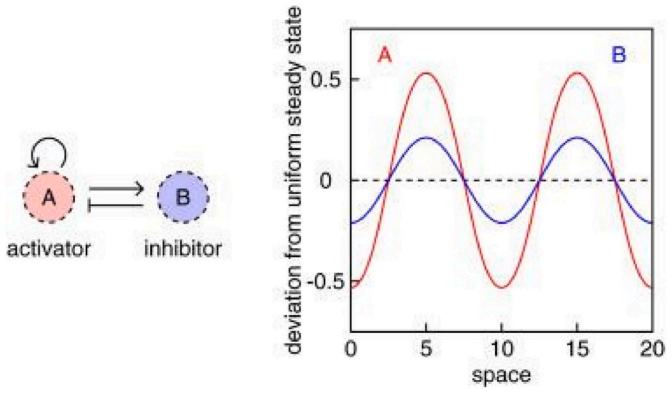

Fig. 2. Schematic drawing of a one-dimensional Turing pattern using two morphogens and 20 cells in a line (Schweisguth and Corson, 2019), with permission of Elsevier. The dashed line is an unstable solution to the equations. Note that Alan Turing himself used periodic boundary conditions (Gordon, 2016; Turing, 1952), which emphasizes that he did not require sources or sinks at the ends.

others (Gierer and Meinhardt, 1972; Kondo and Miura, 2010). For instance, within the framework of Turing RD model, Gierer and Meinhardt provided several important advances (Gierer and Meinhardt, 1972). First, they explicitly pointed out that the primary patterns of morphogens can be generated with only a two-component system including a short-range activation and a long-range inhibition. And Gierer and Meinhardt also found that there are only two possible realizations of the required two components: the activator/inhibitor system and the substrate-depletion model (Gierer and Meinhardt, 1972; Murray, 2011, 2013). Moreover, incorporating realistic nonlinear reaction kinetics and pre-patterng that are often found in developing systems, the Gierer-Meinhardt models obtained robust observed patterns that scale with growing tissue size (Gierer and Meinhardt, 1972). Therefore, although it is difficult to apply the original Turing RD model directly to complex living systems, it has been shown that the general principles underlying the Turing pattern formation may apply to a broad range of real situations (Kondo and Miura, 2010; Roth, 2011). Nonetheless, Gierer and Meinhardt (1972) remains an elaboration of the Turing model with the same limitations for explaining morphogenesis.

It is worthwhile to point out that the word "morphogen" has come into use for any chemical gradients presumed to be related to morphogenesis. Currently, morphogens are considered to be secreted signaling molecules that (i) are generated in a restricted part of a tissue, (ii) are transported by various mechanisms, such as diffusion, motor molecules on cytoskeleton, active transport, and relay mechanisms, to the remainder of the tissue, either through the cells and their junctions or extracellularly, (iii) bind to regulatory regions of DNA or specific receptors, and (iv) initiate an intracellular signal transduction that impacts the expression of target genes in a concentration-dependent manner (Umulis and Othmer, 2015). The Turing reaction-diffusion model does not require a source and a sink (Othmer and Pate, 1980) because each cell is a source and sink. This may be why so many biologists invoking French flag model gradient in morphogenesis, which grew out of Turing reaction-diffusion model, generally ignore the need for both sources and sinks over a whole tissue.

3. The French Flag model

The French flag model was first proposed by Lewis Wolpert in the 1960s as a way to explain morphogenesis (Wolpert, 1968). What Wolpert added to the monotonic gradient idea is that cell differentiation depends on such gradients in a quantitative fashion, i.e., he introduced the idea that a cell can figure out its coordinates in an embryo based on local concentrations of morphogens and act upon them (Wolpert, 1969, 1996, 2000) (Fig. 3). These coordinates thus provide the cell what he called "positional information" (Wolpert, 1969). The two-gradient theory of Leopold von Ubisch (von Ubisch, 1936, 1938, 1952) may have anticipated Wolpert's theory (Marí-Beffa and Knight, 2005).

Thus, in effect Wolpert combined the earlier idea of a monotonic gradient with Turing's concept of cells differentiating in response to a morphogen gradient, to create a one-to-one mapping of a gradient in one direction along one of the three Euclidean orthogonal coordinates: (head to tail, dorsal to ventral, left to right). Note that warping of these three axes by tissue movements was not considered, which is best handled by Lagrangian rather than Euclidean continuum mechanics (Jacobson and Gordon, 1976). Unless diffusion in tissues is anisotropic, the distortions of tissues by movements would also change the directions of each purported gradient relative to a given cell in them, so that they could cease to be orthogonal.

The actual French flag has three colored stripes of equal widths (basically a one-dimensional pattern), each taken as analogous to a specific differentiated cell type. It thus concerns itself with only one axis of the embryo or developing tissue (as, for example, localized development of a limb (Delgado and Torres, 2016, 2017)). In the French flag gradient model each cell measures the local morphogen concentration between a lower and an upper threshold. We can designate these threshold intervals in one direction as (m_i, m_{i+1}), $i = 0, \ldots, n-1$, where $n = 3$ for a tripartite flag pattern. Positional information is thus a "rounding" to the discrete "step" (m_i, m_{i+1}), and determines which cell type that cells in morphogen concentration interval i are supposed to become. Each cell effectively uses its rounded coordinate in a lookup table (Proposition 33 in (Gordon, 1999)) stored in the DNA and responds with the correct, discrete gene expression pattern for that cell type (Wolpert, 1969).

There is no question that concentration gradients do exist in embryos and other developing systems. As we shall discuss here, the major question is whether these are causes or effects of the patterning mechanism. The best-known example is the maternal *bicoid* gradient in *Drosophila* (Ephrussi and St Johnston, 2004; Struhl et al., 1989) which has been the subject of many models (Coppey et al., 2007; Grimm et al., 2010; Kavousanakis et al., 2010; Lipshitz, 2009; Little et al., 2011; Wu et al., 2007; Xie and Hu, 2016). Given the observation of gradients of transcription factors, it became common for embryologists and molecular biologists to speak of a "morphogen gradient" across a tissue, with mathematical biologists providing general models (Dalessi et al., 2012; Kerszberg and Changeux, 1994; Lei and Song, 2010; MacWilliams and Papageorgiou, 1978; Papageorgiou, 1980; Shvartsman and Baker, 2012). The morphogen is released from a site of "induction" and spreads, creating a gradient of morphogens across a tissue. It also requires a sink, either degradation en route or at the opposite boundary, to reach steady state (Chaplain and Stuart, 1991; Conway, 1993; Shostak, 1973; Srinivasan et al., 2002; Zinski et al., 2017), or both. Regeneration of amphibian limbs has been interpreted as providing evidence that cells have positional values (Kumar et al., 2007; Pescitelli and Stocum, 1981).

Some of supporting evidence for the French flag model and the positional information theory is from the patterning and the regeneration experiments in *Drosophila* and other model systems. There are a number of other experimental observations or measurements to provide evidence for the hypothesis that cells have positional values upon which the French flag model and the positional information model were built. A review paper by Wolpert (2011) listed some of them including the anterior-posterior patterning of the *Drosophila* wing imaginal disc via morphogen Decapentaplegic (Dpp), the dorso-ventral patterning of the vertebrate neural tube, gradients in the early *Xenopus* embryo, and pattern formation of the developing zebrafish embryo. For example, in the *Drosophila* wing imaginal disc Dpp is secreted at a strip near the anterior-posterior compartment boundary and considered as a long-range morphogen to control patterning and growth through forming a concentration gradient across the wing disc (Tabata and Takei, 2004). In the ventral neural tube Sonic hedgehog probably

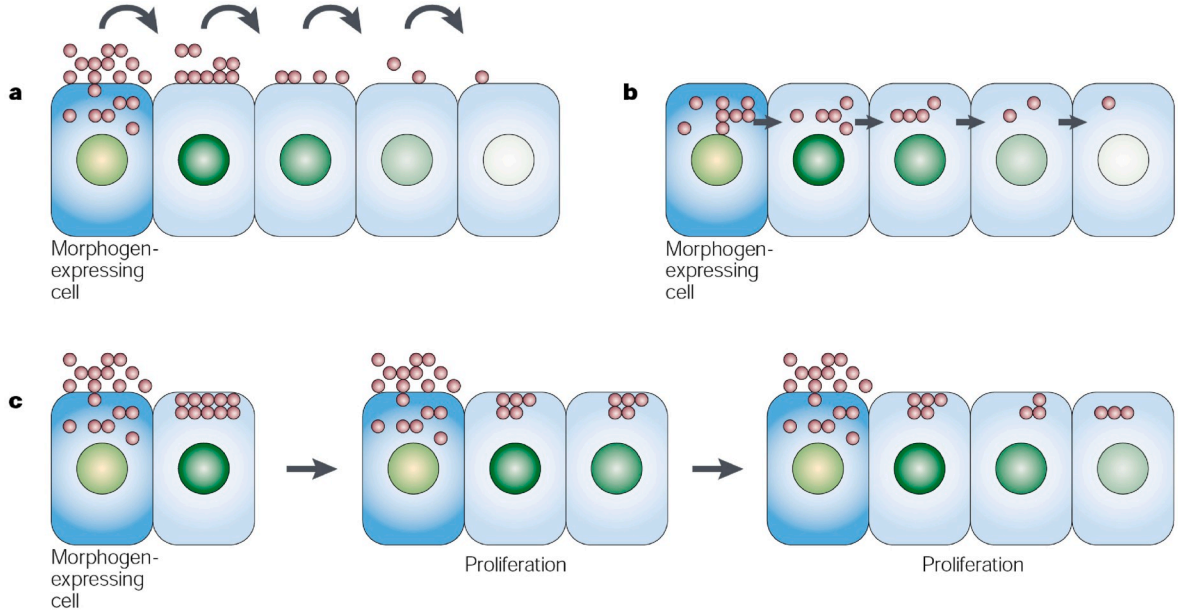

Fig. 3. "Three models for the generation of gradients of molecules presumed to be 'morphogens', i.e., molecules that cells hypothetically use to decide, according to the concentration of the morphogen, whether or not to differentiate. a) Diffusion through the extracellular space. b) Planar transcytosis. c) Displacement during growth" (Gordon and Gordon, 2016a). From (Tabata, 2001) with permission of Springer Nature.

provides a signal gradient (Dessaud et al., 2010); BMP4, which acts as a morphogen in the early *Xenopus* embryo, is believed to pattern the dorso-ventral (DV) mesoderm and neuroectoderm in a concentration-dependent manner (Niehrs, 2010). Moreover, in the zebrafish embryo, it is thought that molecules of the nodal family form a morphogen gradient that guides pattern formation. Cells experiencing high levels of nodal signaling develop into mesoderm while cells become ectoderm when they sense low concentrations of nodal signaling (Harvey and Smith, 2009).

The French flag model has received quite a bit of theoretical attention, including attempts to achieve the same pattern by other mechanisms (Aguilar-Hidalgo et al., 2015; Bakowska et al., 1982; Benazet and Zeller, 2009; Bowers, 2005; Chavoya et al., 2010a, 2010b; Chavoya and Duthen, 2007, 2008; Denetclaw and Ordahl, 2000; Devert et al., 2011; Gunji and Ono, 2012; Herman, 1972; Herman and Liu, 1973; Hillenbrand et al., 2016; Jaeger, 2009; Jaeger and Reinitz, 2006; Joachimczak and Wrobel, 2012; Knabe et al., 2010; Lindenmayer and Rozenberg, 1972; Liu et al., 2005; Lynn and Tucker, 1976; Miller, 2003, 2004; Miller and Banzhaf, 2003; Othmer and Pate, 1980; Pecze, 2018; Pont et al., 2016; Quininao et al., 2015; Sarr et al., 2014; Sinner et al., 2015; Sutantyo et al., 2016; Tautu, 1975; Wolpert, 1968, 1969, 1970; Wolpert et al., 2019; Woolley et al., 2011; Xiong et al., 2013; Xu et al., 2012; Zadorin et al., 2017). It, together with morphogen gradients, has become the accepted model of embryogenesis, widely mentioned in textbooks and monographs across many disciplines (Arthur, 1987, 1988; Baltimore, 2002; Chauvet, 1996; Furcht and Hoffman, 2008; Gray and Williams, 1989; Heming, 2003; Ho and Saunders, 1984; Luo et al., 1997; Marí-Beffa and Knight, 2005; Nadel, 2003; Ord and Stocken, 1998; Purves and Lichtman, 1985; Rose, 1998, 2005; Saunders, 1982; Smith and Wood, 1992; Smith and Szathmary, 2000), with only a few expressing any doubts (Held, 1992; van der Wal et al., 1997). It has even been discussed in at least one newspaper (Anonymous, 2000) and has penetrated the public via popular books (Carroll, 2005; Wolpert, 1991). "Despite a huge literature on morphogens, even Lewis Wolpert, who coined the phrase in 1969 (Wolpert, 1969), has expressed doubts about the reality of positional information (Kerszberg and Wolpert, 2007; Wolpert, 2011)" (Gordon and Gordon, 2016a).

This widespread acceptance of the French Flag Model grew out of a confluence of factors. First, classical embryology was originally taught as a subset of anatomy with heavy emphasis on the four-dimensional nature of the embryo. The embryo begins as a single cell and transforms over time into many cell types in somewhat precisely replicated positions and forms, which had to memorized. Unlike studying the anatomy of an adult where an organ such as the heart is fully formed and can always be found in the same position, in an embryo everything constantly changes with time. The result is a tendency to look at an embryo at one point in time and ignore the rest of development either before or after that point, particularly if that point in time contains a measurable gradient of something to which subsequent development can be attributed. This attitude was further reinforced by a dramatic switch in embryology when the classical anatomical approach was largely replaced by the molecular biology approach. Classical anatomical embryology is no longer widely taught outside of medicine and engineering (Gordon, 2013; Gordon and Melvin, 2003). The entire field of molecular developmental biology is based on the premise that since different cell types express different subsets of genes, everything should be understandable by figuring out the gene networks and gene expression patterns, with the implicit assumption that mathematical biologists have justified this approach. In 1970 Francis Crick showed that diffusion gradients could be established on the time scale of embryogenesis in a *Nature* paper that at the time of this writing has 618 citations (Crick, 1970). The subsequent discovery of gene gradients that he called for seemed to prove the idea that something creates gradients which creates changes in gene expression in a causal, gradient-based manner. Due to lack of a better model, the French flag gradient model has persisted.

There are still unsettled central issues with the morphogen gradient model, the positional information model and the French flag model, despite their popularity. Still under investigation or under debate are how positional information is set up, how it is recorded, and then how it is interpreted by the cells (Wolpert, 2011). Limited information exists on how gradients are formed. The diffusive mechanism, coupled with the uptake by cell-surface receptors and subsequent degradation, is considered as the most plausible explanation for how morphogen gradients are generated at the level of the DNA (Lander et al., 2002). However, whenever doubts about the functioning of these so-called "morphogen" gradients have been raised (including by Wolpert (Beloussov and Gordon, 2018; Kerszberg and Wolpert, 2007; Richardson and Wolpert, 2009; Wolpert, 2011; Wolpert, 2017)), alternatives,

elaborations, and transport mechanisms other than simple diffusion are proposed including endocytosis and transcytosis or even anthropomorphic concepts like "bucket brigades" (Chen and Zou, 2019). Second, in spite of the popularity of the model, these concepts remain unproven even though gradients undoubtedly exist. Numerous molecules have been proposed to be morphogens (Hiscock and Megason, 2015; Nüsslein-Volhard and Wieschaus, 1980). As new biologically active molecules are discovered, they are often added to the list (Inui et al., 2012) and then later sometimes removed (Franceschi, 1992). There is no good evidence for the quantitative analysis of any reported gradients, and there is no molecular basis of the positional values available in any system (Wolpert, 2011). Furthermore, mechanistic issues were raised with the idea that a signaling gradient specifies differential gene expression in a concentration-dependent manner which involves threshold and temporal effects (Wolpert, 2011). Therefore, although there exist diverse proposed mechanisms and models for morphogen-mediated patterning, lack of quantitative measurement of gradients and limited knowledge on how gradients are built and explained remain a consistent problem.

There are further problems with the French flag gradient model (Gordon and Gordon, 2016a):

1. The speed of development may not permit steady state to be reached (Berezhkovskii et al., 2011; Bergmann et al., 2007; de Lachapelle and Bergmann, 2010a; Yin et al., 2013). This is sometimes considered an advantage in cases where the steady state could not possibly lead to the correct morphology. So we have a steady state invoked except when we don't want it (Bergmann et al., 2008; Saunders and Howard, 2009). In any case, the rate of development varies substantially with temperature over a species' temperature range for normal development (Bachmann, 1969; Duellman and Trueb, 1986; Volpe, 1957). This would have to be matched to the temperature dependence of diffusion of the molecule (Cussler, 2009) which is itself dependent on the temperature variation of the viscosity of the medium through which the molecule diffuses (Seeton, 2006).
2. Ordinary diffusion gradients do not scale well (Barkai and Shilo, 2009; McHale et al., 2006). The consequence is that for embryos of different sizes there should be widely different proportions of parts, but we know that is not the case (de Lachapelle and Bergmann, 2010b). There is a limit on the "range" of a morphogen gradient (Kanodia et al., 2011). This limits their potential role in growing tissues (Hamaratoglu et al., 2009; Yin et al., 2012). Amphibian embryo eggs vary from 0.75 mm to 35 mm in diameter (Table 2 in (Tuszynski and Gordon, 2012)), and yet produce adults with substantially the same body plan. As we have a common ancestor with amphibians, our own eggs at 0.07 mm extend the diameter range down by another order of magnitude. Scale independence requires that diffusion, reaction rate, and/or source intensity be manipulable by the embryo (Umulis and Othmer, 2015).
3. The fundamental principle of gradients is that cells in high concentrations will respond in one way, while those at low concentrations respond in a different way, while those in the middle respond in yet another way. Fluctuations in gradients always occur, especially if the number of diffusing molecules is low. Fluctuations of purported morphogen concentrations make response to particular concentration thresholds problematic (Eldar et al., 2002; Morishita and Iwasa, 2009; Wu et al., 2007).
4. Each cell has to be able to "read" the morphogen concentration accurately (Bothma et al., 2010; Gurdon and Bourillot, 2001; Kerszberg, 1996, 1999; MacWilliams and Papageorgiou, 1978; Tamari and Barkai, 2012), lest boundaries between tissues become ragged (Emberly, 2008). Gradients are frequently invoked without any explanation of how a cell measures a concentration. Yet in embryos boundaries between tissues are generally sharp, at the cellular level.

There is also widespread misunderstanding by biologists of Alan Turing's (Turing, 1952) reaction-diffusion equations which can set up spatially or temporally periodic patterns. These patterns in themselves appear incapable of explaining more than one step of cell differentiation (Gordon, 2016). It is generally ignored that Turing invoked both chemical and mechanical instabilities (Turing, 1952; Vilaca et al., 2019). Note that pattern formation by mechanical instabilities goes back to Lord Rayleigh's work on drop formation from "one-dimensional" cylinders of fluid (Rayleigh, 1879a; b, 1892), which we have applied to cell sorting (Gordon et al., 1972, 1975). Mechanical instabilities have also been proposed as the basis for feather spacing patterns (Murray and Oster, 1984b; Perelson et al., 1986), with reaction-diffusion patterns claimed to be a subset of those generable mechanically (Murray and Oster, 1984a). The French Flag model works solely by chemical diffusion ignoring any mechanical component. The Embryonic Differentiation Waves Model including both mechanical and chemical components suggests the possibility of an underlying theory encompassing both differentiation waves and reaction-diffusion (§1.15 in (Gordon, 1999)).

4. The Embryonic Differentiation Waves Model

A mechanochemical model for cell differentiation based on differentiation waves was first proposed in 1987 by Gordon and Brodland (1987). (The clothesline model (von Uexküll, 1926) may have anticipated differentiation waves (p. 36 in (Gordon, 1999)).) The Gordon and Brodland model uses a mechanically sensitive bistable organelle made of microtubules and microfilaments (Burnside, 1971, 1973; Gordon and Jacobson, 1978; Jacobson and Gordon, 1976) that occurs in the apical ends of cells within cell sheets when they are ready to differentiate. This orgnanelle is called the cell state splitter (Björklund and Gordon, 2006; Gordon and Gordon, 2016b; Gordon and Brodland, 1987). Competent cells are under mechanical tension with the microtubule mat and microfilament ring in radial mechanical opposition, metastabilized in most cells by an intermediate filament ring (Martin and Gordon, 1997). Depending on where the cell is within a sheet, the tension is resolved by its apical end either contracting or expanding, a binary response. The resolution of the instability begins at one point with an "organiser" consisting of a cell or small subset of cells experiencing a mechanically induced contraction and a different cell or small subset of cells at a substantial distance in another place experiencing an expansion. Once a wave begins, the contraction or expansion wave, which is visible in time-lapse microscopy (Crawford-Young et al., 2018; Gordon and Björklund, 1996), is propagated to adjacent cells (Fig. 4). Halting of wave propagation may involve mechanical forces at boundaries, or the propagating wave reaching cells that do not have their bistable cell state splitter ready to respond. An actual physical wave of contraction was found that traverses the presumptive neural epithelium of the developing salamander, the axolotl (*Ambystoma mexicanum*) in 1990 (Figure 59 in (Gordon, 1999); (Brodland et al., 1994)). It is 0.1 mm wide and deep on this 2 mm diameter embryo (Gordon and Björklund, 1996). Additional waves were then discovered on the axolotl embryo (Gordon et al., 1994), although not in the South African clawed toad *Xenopus laevis*, perhaps due to an overlying superficial epithelium (Nieuwkoop et al., 1996). The trajectory of each wave corresponds to differentiation of a different classically defined embryonic tissue (Gordon et al., 1994). Waves can begin at a point and expand outward, initiate along a line and travel as a moving furrow, or begin as a circle moving inward, depending on the mechanics of the cell sheet within the embryo as a whole. Entire sections of cell sheets can be observed contracting as a unit (Gordon and Gordon, 2016a). Note that the morphogenetic furrow of the *Drosophila* eye imaginal disc can be interpreted as a differentiation wave (Alicea et al., 2018; Gordon, 1999).

The trajectories of contraction and expansion waves were superimposed on the axolotl fate map, which illustrates developmental anatomy of the axolotl over time (Cleine and Slack, 1985; Piekarski and Olsson, 2007; Vogt, 1925, 1929), in (Björklund and Gordon, 1994; Gordon et al., 1994). This revealed that there is a unique bifurcating

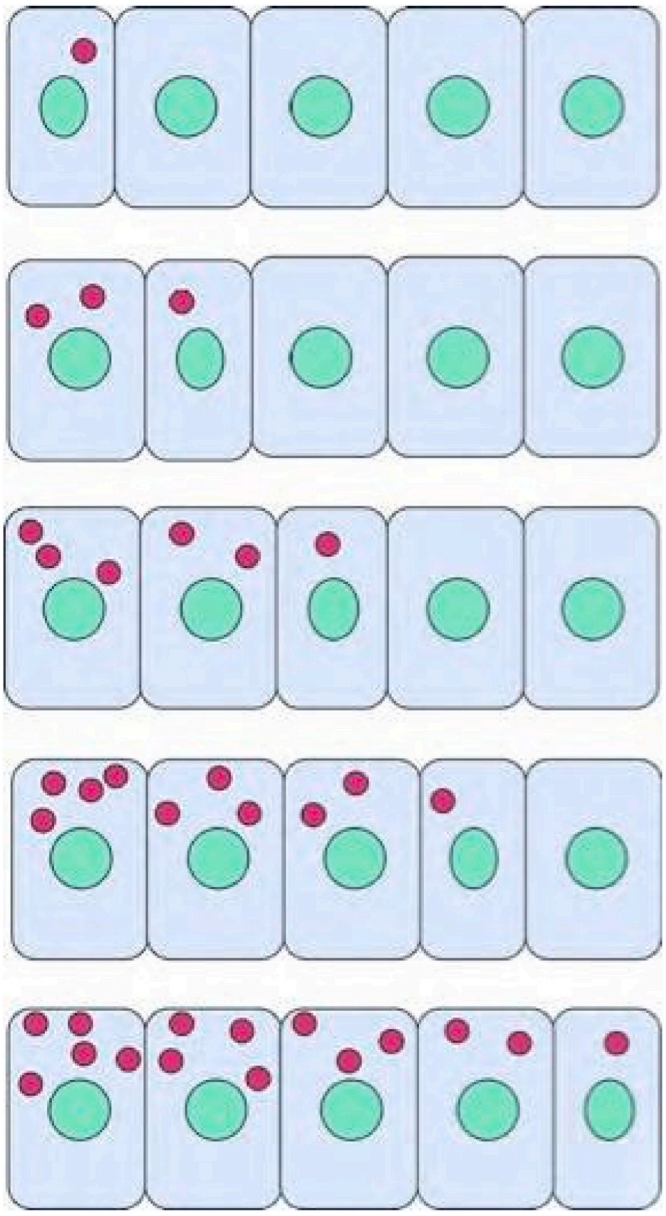

Fig. 4. "A contraction [differentiation] wave is depicted propagating from one cell to the next in an epithelial layer of cells. This initiates differentiation of the cell, which starts to produce cell type specific molecules, shown in red. If these molecules increase in number over time, as the wave propagates, a gradient of the molecules will develop across the epithelium, especially because differentiation waves propagate slowly, taking hours to cross it. The differentiation [or at least commitment] has already occurred, so the gradient is not causal of differentiation. In fact, the gradient may be called an irrelevant epiphenomenon" (Gordon and Gordon, 2016a), with permission of World Scientific Press. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

sequence of expansion and contraction waves (the "differentiation tree" (Alicea and Gordon, 2016; Martin and Gordon, 1995)) that correlates with tissue types determined up to neural tube closure (Björklund and Gordon, 1994). The binary nature of the branches of the differentiation tree may be represented as a differentiation code (Gordon and Gordon, 2016a, 2019; Gordon, 1999), providing a discreteness different from that of the French flag. One of two readied signal transduction pathways from the cell state splitter to the nucleus results in changes in gene expression (Björklund and Gordon, 1993; Gordon and Gordon, 2016a). There are two pathways, one for contraction, the other for expansion. This pair of pathways amounts to a one-bit signal from the cell state splitter to the nucleus.

Each of the signal transduction pathways used in the cell state splitter model consists of multiple elements such as *wnt*. These are commonly invoked as morphogens in the French flag model, but their functions in the cell state splitter model are as components of the contraction or expansion signal transduction pathway, active during the change of state of the cell. The initial phase is classically called "commitment" or "determination" to a later "differentiated" state. All the other activity, such as changes in gene expression, signaling proteins like *wnt*, release of additional morphogens, and epigenetic changes, are the result of commitment/determination and subsequent differentiation of the cell after the response of the cytoskeleton to mechanical signals (Gordon and Gordon, 2016a). As these molecules appear or are activated when the individual cell contracts or expands its apical surface, their concentrations will vary across the tissue containing that cell. In other words, a differentiation wave generates one or more gradients as it travels through a tissue, and the gradients thus may be regarded as epiphonema subsequent to wave passage. Cells in the cell state splitter model require no more than an epigenetic mechanism for keeping track of the number of contraction and expansion waves they participate in, which can be based on well documented mechanisms such as changes in HOX genes as tissues differentiate (Papageorgiou, 2014).

According to the cell state splitter model, embryonic differentiation does not occur due to gradients. Embryonic differentiation is temporally and spatially directed by biochemical/mechanical/ion-electrical differentiation waves in an active medium, a sheet of cells. The mathematics applicable should be that of activation waves. These are solitary "kink waves", also called "front waves" (Gordon, 1999; Kuramoto, 1984). They are not ordinary superimposable waves nor solitons (Scott et al., 1973), both of which can pass through one another unaffected. Common examples of kink waves are propagating phase transitions or fires. Differentiation waves are kink waves because they cause a change in cell type.

The passage of the differentiation wave will produce a temporary gradient of cytoskeletal rearrangement, signal transduction and gene expression strictly as a by-product. If the differentiation wave begins at a boundary and travels away from it, higher levels of specific gene expression can be expected at the boundary zone as it experienced the differentiation wave sooner and has had more time to up regulate production of the specific gene products being measured. The reverse would be true with down regulation of a specific gene product. By changing the model on which the mathematical assumptions are based from morphogen diffusion to differentiation waves this new model may resolve many of the troubling aspects of attempting to do the mathematics of differentiation using diffusion based models. This includes the rises in the boundary regions of differentiating tissue reported by Chen and Zou (2019). We therefore propose that the cell state splitter model of embryonic differentiation waves be tested against the French flag model by the ability to correctly predict spatiotemporal gradients. If the differentiation wave model is correct such testing will match observations in living embryos while the French flag model will fail to do so. The differentiation wave model predicts that gradients should follow after initiation of participation of a cell in a differentiation wave, and thus reflect the trajectories of the waves, which are generally not straight lines, making this a robust prediction. There is still a critically important role for the study of gradients in embryonic development: gradients can be used to plot the presence and trajectories of cell state splitter differentiation waves. In classical embryological terms, passage of a differentiation wave through a cell determines its fate, while the gene products generated cause its differentiation to a new cell type.

5. Conclusion

The story of differentiation waves versus morphogen gradients is far from over, with at least one call to test their relative success in modelling

embryogenesis via computer models (Miller, 2017). All three models, gradient French flag, embryonic differentiation waves, and Turing reaction-diffusion claim to explain what causes a change of state of the cells from one cell type to another (Figs. 2–4). As we have noted, in the plethora of models are some that combine models, as in the "global wave" with local Turing effects of (Inaba et al., 2019) (which is equivalent to "Type 2 Cell sheet alternating differentiation wave" in (Gordon and Gordon, 2016a), without invoking a Turing mechanism). All three models propagate in space, changing the state of the material they pass through, and are therefore examples of kink waves (Gordon, 1999; Kuramoto, 1984). In all three approaches to explain embryogenesis, the spatial and temporal construction and control of boundary conditions has hardly been explored.

The embryonic differentiation wave model assumes the whole process can be regarded as a bifurcating sequence of construction and execution of cybernetic control systems (Gordon and Stone, 2016). The goals of each control system are to change the state of differentiation of subsets of cells and establish the conditions for generating the next pair of control systems.

Insofar as each embryonic differentiation wave has mechanical, chemical and ionic components (Gordon, 1999), it might be a means by which global controls of embryogenesis occur, with some waves traversing more than one embryonic tissue, as has been observed in axolotl embryos (Gordon and Stone, 2016). This may provide a consolidation of global bioelectric observations in embryos (Mathews and Levin, 2018; Pietak and Levin, 2018) with embryonic differentiation waves. There are many opportunities here for mathematical biologists to suggest critical experiments and predict their outcomes, in a hopefully convergent cycle: experiments and observations, formal mathematics, and computer simulations (Jacobson and Gordon, 1976). The embryonic differentiation waves potentially represent a paradigm shift (Barresi and Gilbert, 2020; Miller, 2017; Papageorgiou, 2001). As such, it will probably be ignored until the contradictions and elaborations of gradient models become obvious to newcomers to the field (Kuhn, 1996). Perhaps that time has come.

It has been understood since at least Charles Darwin's time that observation is driven by theory: "I am a firm believer, that without speculation there is no good and original observation" (Darwin, 1887). By noting that the French Flag gradient model is not the only model for cell differentiation, we hope that the development of theory can be advanced, so that observations relevant to distinguishing the embryonic differentiation wave model from the French Flag model and reaction-diffusion equations will be taken up by experimentalists. Experimentalists rely on mathematical biologists for theory, and therefore depend on them for what parameters they choose to measure and to ignore.

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