Commentary   **Open Access**

Re-thinking the Networks

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Introduction

Signalling perceptions

Enormous strides have been made since the recognition of receptors (initially in the surface membrane) which interact with a specific molecule and somehow transmit a signal to a cell to do something ("stimulus-response" coupling). This response often centred on inducing proliferation from a resting state or secretion of a particular molecule, and was followed by molecular characterisation of growth factors and their cognate receptors. Further dissection of responses established intermediates whose function is to interact in sequential biochemical reactions, thus forming a "chain". In parallel, the blossoming of immunology and molecular embryology revealed not only "signalling" in individual cells, but a whole series of regulated cell interactions (T-cells, B-cells, macrophages/antigen-presenting cells, the creation of the thymic T-cell repertoire and the B-cell antibody library; in embryology, stages regulated by timed expression of transcription factors and surface receptors). In addition, therefore, to signalling being a concatenated series of intracellular biochemical events, signalling *between* cells became part of the scene at the cell-cell level inextricably linked to the former.

The principal observation is that a unitary initial stimulus provokes responses which are far more global; the response "spreads out" within and between many other areas/systems. So there must be (potential) "signalling networks" into which the initial stimulus fits. By extension, if the initial stimulus is identified then any part of this network can be targeted for modification. Therapeutic agents therefore have two goals: cell specificity; and ability to target with precision any focal point considered the origin of (defect in) cell (dys) function. Cell specificity is a separate issue but clearly the expression of cell phenotype is determined by signalling networks and herein lie some problems.

The Current Signalling Concept

Figure 1 presents a stylised diagram of a signal transduction pathway, in this case generically dealing with cancer induction (reproduced from reference [1]). This article addresses the issue that while the "signalling" agents inducing cancer are extremely variable, cancer cells all express the same progressive attributes. Signalling diagrams provide a description of events as a linear array, the arrows indicating what reacts with what, a temporal/positional sequence, potential checks and balances and the idea of a "forward drive", ie., given an initial event, in this case a primary mutation, a set of reactions takes place which drives the whole "network" to a conclusion. The inference is that given knowledge of the detail in each signalling pathway, we can sequentially dissect "systems", e.g., "growth", "gene expression", "immune recognition", "metabolism" etc., and thus understand how different signalling systems fit together, for example to explain cancer.

Many, many diagrams of signalling can be drawn dealing with many specific receptors and observed responses at various levels of detail. In fact, they all look quite the same which will be referred to later. One only has to remember the highly informative BDH wall chart of intermediary metabolism of some years ago needing an arrow saying "you are here" to establish just where to start; signalling provides this initial event.

One might therefore expect that the knowledge base would indicate progressive integration of different pathways as more information arises, and the numbers of publications would begin to level off as their connexions are established. A brief, limited survey shows the opposite in Table 1.

While the search terms included are quite general, these are huge numbers. Nearly all have been published over the last 10 years, and at least 60% in the last 5. Even if only 80% present one new observation, the information content, which can only be extracted for relevance by reading it (context-limitation), is virtually unmanageable.

Realities

Signalling concepts are based on observable facts from scientific experiment, are highly informative, explain sequences of molecular events and provide avenues for further exploration. And, there is no question that they are predictive, ie., cells will respond to signals in a way that can often be predicted overall, e.g., secretion of histamine on IgA binding, proliferation of T cells with IL2, and induction of transformation by a single entity such as mutated ras. However, how do the derived realities of signal transduction correspond with what happens in a cell?

Restriction and variation in response

In experimental cell systems account has to be taken of variation in response (reproducibility) according to often unquantified conditions. Thus, in transfection of cells with a potent mutated oncogene e.g., ras only a proportion of cells will be transformed. In drug trialling, responses will vary between batches of even the "same" cells. "Side-effects" are a major part of interventions and while often related to drug metabolism, they are actually indicating different signalling pathways in other cells.
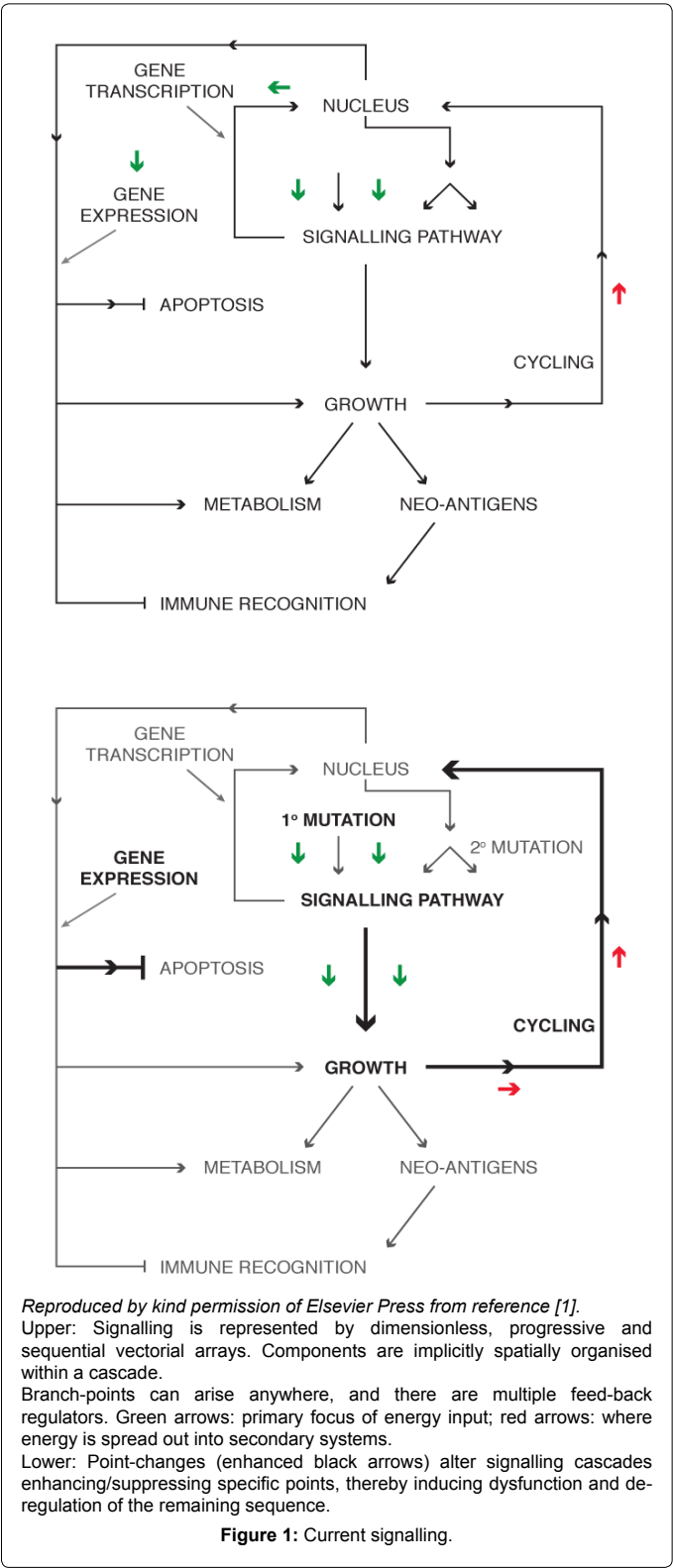
Scale and distance

If the dot over the 'i' in "in" represents the molecular dimensions of a surface receptor and signalling intermediates, the distance the signal needs to travel to the nucleus would be about 1 meter. The components of cytoplasmic intermediates per pathway usually number around six to ten, so the interaction travel distance per molecule is still very large. Passive diffusion is most unlikely and problematic for large irregular molecules like proteins in complex mixtures such as cytoplasm. A major question is how do intermediates find each other? None are at saturation densities and random events therefore seem unlikely too. Clearly, formation of dynamic structure (self-assembly/organisation) must play an important role in providing multi-molecular "hub"

Reproduced by kind permission of Elsevier Press from reference [1].
Upper: Signalling is represented by dimensionless, progressive and sequential vectorial arrays. Components are implicitly spatially organised within a cascade.
Branch-points can arise anywhere, and there are multiple feed-back regulators. Green arrows: primary focus of energy input; red arrows: where energy is spread out into secondary systems.
Lower: Point-changes (enhanced black arrows) alter signalling cascades enhancing/suppressing specific points, thereby inducing dysfunction and de-regulation of the remaining sequence.

Figure 1: Current signalling.

assemblies in which movement is integral to construction; this is dealt with later concerning emergent properties in complex systems.

Space-filling

The signalling diagram assumes empty space and close proximity of

intermediates. However, the internal cellular environment is extremely diverse and packed to near-saturation with molecules in solution or suspension, together with structural components such as cytoskeleton, membranes, molecular complexes etc., collectively termed "molecular crowding" [2]. All obstruct free movement. Of course, there are energy-activated motor and cargo-carrying systems but their use requires guidance, first for a molecule to reach an available cargo carrier, and second to direct the complex to a specific target. Bearing in mind the potential proteins available for carrying, there has to be a mechanism for selecting "appropriate" ones on signal initiation.

Quantitation

At present, most signal transduction is relatively unquantified. Thus, we usually do not know how many surface receptors there are, the occupancy needed for a response, the concentration, distribution and equilibria of interaction of each intermediate, and the context of the experimental signal, i.e. how many other potentially competitive signalling pathways are in existence at the same time in the same vicinity. Of course, signals are amplified because one enzyme can generate a lot of product, but usually there is no information on the degree of amplification at each step, nor where intersection is possible and what decides any thresholds other than simple concentrations of reactants (see below on molecular crowding). These parameters are definitive to understand how molecular chains functionally organise.

"Spreading" the signal: intersections/branch points in a network

Clearly, pathways intersect with others because as mentioned signalling most often is accompanied by multiple effects in other highly complicated systems such as replication control, cell movement, and protein expression. Conceptually this is dealt with by creating networks where different pathways converge or have branch-points to intersect. However, each branch point generates uncertainty; there is a "choice" between possibilities of reactions with other network components. As the numbers of different networks are uncovered, the higher is the uncertainty for any one to reach a particular or predicted outcome. And, at the final analysis, every pathway is ultimately connected to every other one because cells are finite systems which are dynamically stable, albeit within limits. Signalling therefore reduces to the most favourable outcome at the time rather than an instruction to infallibly produce a specific outcome; the less interconnected the pathway, the higher is the probability of a consistent response. The problem is that intersections in linearised networks cannot effectively deal with multiple and unpredictable variations in the network due to lack of information on the dynamics of component interactions.

Heterogeneity within signalling pathways

Signalling diagrams appear as definitive maps, giving the impression that this map is duplicated anywhere that signal is applied and is invariant. However, there may be very many origins where the same signal is applied, but the environment of each individual pathway

Search terms included	No. of publications	% from last 10 years
signalling	583,222	99.9
cell signalling	518,422	99.9
signalling network	19,002	99.9
cell signalling network	102,060	75

Data retrieved from PubMed.

Table 1: A brief, limited survey.

Somatic genes
AHNAK
AKT1
BMP1
BMP2
BMP7
CALD1
CAMK2N1
CAV2
CDH1
CDH2
COL1A2
COL3A1
COL5A2
DSP
EFGR
ERBB3
FGFBP1
FN1
F11R
F2D7
GNG11
GSC
IGFBP4
ILK
IL1RN
ITGAS
ITGAV
ITGB1
JAG1
KRT14
KRT19
KRTZ
MAP1B
MMP2
MMP3
MMP9
MSN
MST1R
NUDT13
NODAL
OCCN
PDGFRB
PLEK2
PPPDE2
PTK2
PTP4A1
RAC1
RGSL
SERPIN1
SPARC
SPP1
STEAP1
TGFb1
TGFb2
TGFb3
TIMP1
TFP12
TMEFF1
TMEM132A

TSPAN13
VCAN
VIM
VPS13A
WINT5A
WINT5B
WNT11
Transcriptional regulators
CTNNB1
FOXC2
NOTCH1
SMAD2
SNAIL1
SNAIL2
SNAIL3
SOX10
STAT3
TCF3
TCF4
TWIST1
ZEB1
ZEB2

Data from Qiagen PLC and other sources.

Table 2: Genes involved in EMT.

or component may be very different. The signalling diagram is thus more of a consensus of results and the question then arises as to how any signal is given preference over the *status quo*. Thus, while specific and consistent signalling can be observed, as one focuses down onto a singular one its uniqueness disappears into "black boxes", i.e. complex systems such as "proliferation", "DNA replication". That apoptosis signalling appears definitive is because its components activate small numbers of irreversible enzyme-induced degradations which dismantle everything. For example, cytochrome c release and activation of the caspase 9 axis are dependent on mitochondrial physiology but the mechanisms inducing this signalling are very varied.

The Data Mountain; Processing and Integration in Molecular Genetics

Molecular genetics has made incredible strides in uncovering molecules involved in signalling, how they interact and regulate intracellular activities. Such observations produce data which requires integration, and in general they are inserted into schemes of signalling pathways and proposed networks. However, as with quantitation above, the more mutations/regulators etc., are uncovered, the more is the uncertainty as to precisely how they relate to each other. Two examples may be illustrative.

Epithelial-mesenchyme transition

This is where differentiated epithelial cells undergo a change to a different phenotype (mesenchymal) [3] which bears the hallmarks of certain behaviour in cancer cells, notably loss of epithelial differentiation markers and increased motility and invasiveness. Intensive research has identified over 60 somatic genes and 14 transcription factors associated with EMT, Table 2. The somatic genes cover many different signalling pathways and proteome cassettes; and each transcription factor may regulate (positively or negatively, or both under certain conditions) expression of multiple proteins. Working out possibilities for how they

"network" to produce a given observed change such as EMT is really not possible.

Oncogenes

There are some 200 known oncogenes, i.e. those where a mutation experimentally induces cell transformation or occurs with significant incidence in known cancers. They cover elements in virtually the entire proteome/transcriptome spectra. While the search for new ones or new signalling intermediates/pathway intersections justifies incremental knowledge from which relationships may potentially be extracted and simplified, a relatively straightforward search shows that over 120,000 publications have been generated from just 10 from the oncogene list over the last 10 years.

In both these cases it is beyond present computing capacity to analyse all potential interactions or determine significance of single elements within the whole; the numbers are astronomically large even if (in the case of publications) a new observation is set within the known others considered to be significant. The result is a data mountain which cannot be effectively dissected because there is too much potential for relationships and uncertainty in significance overall. Nevertheless, cells patently manage everything supremely well.

Experimental Systems

Using cultured cells has been supremely successful in elucidating signalling, and indeed it is the only real way of generating a controllable context. Discussion on *in vivo* versus *in vitro* is therefore pointless except obviously that the cells *in vitro* are not the same as *in vivo*. Much is self-evident, for example all mammalian cells require cocktails of growth factors in culture even to survive. Reproducibility relies on carefully limiting variables but in a real context the variables exist, so which are the "correct" ones? Again, there is growing interest on an apparent "switch" from oxidative to non-oxidative metabolism in cancer cells (the Warburg effect) but many cells in tissue culture used in signalling studies express non-oxidative metabolism to varying degrees, even to completeness although the environment is normoxic. More so, many cells used are adherent and there is extensive evidence that the substrate profoundly affects cell behaviour: surface topography, charge distribution and density, adsorbed proteins, all significantly influence shape, motility, expression of matrix genes etc., so any specific experimental signalling is welded into a context.

Re-thinking Signalling

Paradoxically, while signals are demonstrably networked, the more pathways and intersections are found, the more difficult it is to unravel them. What is required is a mechanism by which huge numbers of co-existing molecules and signalling pathways in crowded environments can not only find appropriate interactive partners but can create highly individual series of interactions which somehow create integrated, focussed dynamic activity and an amazing variety of co-ordinated structures, i.e., "emergent properties" where qualities arise from interactions which cannot be foreseen, see later.

It is not by chance that signals "spread out". The guiding physical laws for biochemical reactions include those of thermodynamics, particularly that of Entropy - the degree to which energy is dissipated (i.e., "spread out") in any energy exchange. If, instead of regarding signalling pathways as directional links between closely-proximal, unquantified biochemical point-reactions, they are viewed as maps of energy exchange and dissipation, then components need not physically form a linear chain in close apposition (which cannot be

the case anyway); they can be contained within facilitatory entropic environments which are entirely fluid. Components move with entropic flows which continuously change as energy within them is dissipated through enclosed reactions. During these, components meet, then separate and move independently because of entropic constraints.

But this cannot be random otherwise all becomes unworkable – there has to be dynamic organisation. The second re-think concerns the fact that all signalling diagrams and networks look much the same regardless of scale. Such organisation has all the attributes of Fractal distributions, in which the same overall pattern is repeated at all levels. Furthermore, the properties of fractals allow seamless merging at all levels.

If these are put together into a dynamic format then many of the reality problems can be minimised. Entropy drives molecules to facilitatory environments, and the fluidity of the "map" allows rapid changes at all levels. Because these flows are fractal, they can merge and be duplicated at every level so that individual reactions can occur simultaneously and transiently at multiple sites. Further, the physical properties of entropic systems allows for quite extra-ordinary movement and selection. The spreading out of signalling can therefore be thought of as simply a reflection of how energy itself is being "spread out", i.e., its entropic "map". This map can be different from a signalling map because the latter only indicates sequences of interactions; where they happen has to be represented by boxes, "hubs", open spaces etc. An entropy map, however, defines where such interactions become energetically possible in crowded spaces and how interactions can be managed.
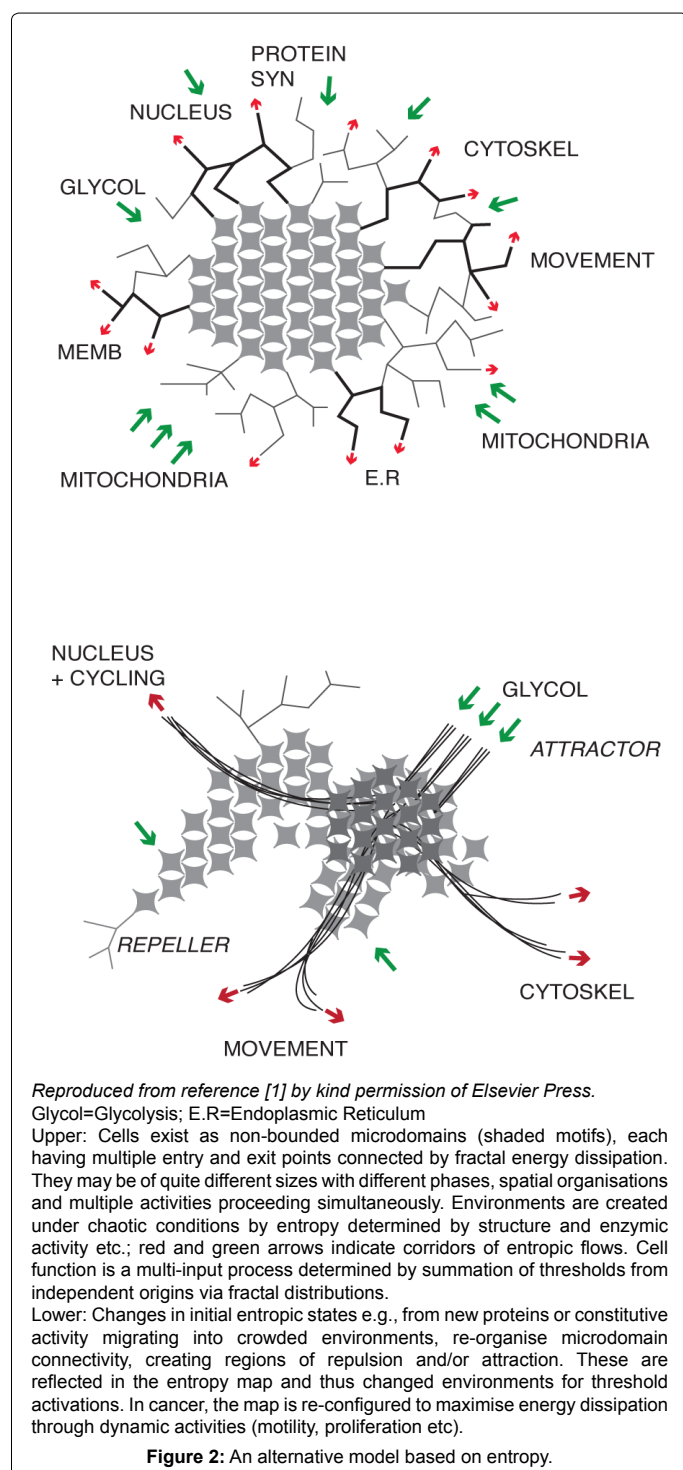
These considerations have been formulated into a model of Fractal Entropy applied to the origin of cancer (reference [1] and Figure 2). In Figure 2, signalling is presented as amalgamation of entropic "corridors" derived from micro-domains. The dynamic behaviour of entropy "maps" is illustrated by Mandelbrot figures (Figure 3) where shapes seamlessly metamorphose into others of the same profile, thus creating many signalling environments simultaneously ([1] contains a link to a video clip of this metamorphosis). It also proposes that origins of signalling are chaotic, i.e., highly dependent on, but not proportional to, initial conditions. Chaos can create fractal conditions influenced by external conditions, and Figure 4 (also in a video clip) shows how fractal origins can be deviated by chaotic environments. Between them they allow for creation of enormous variety in conditions for seamlessly integrating many, many different "pathways" while still maintaining contexts overall; the basic "ground plan" is inherently stable while dynamically very fluid.

In the FE model, cancer is a re-profiling of the entropy map to maximise energy dissipation by diverting it into activity (motility, proliferation etc.), induced by insertion of constitutive activities into chaotic origins. Since there are very large numbers of these, many modalities can have a similar effect; hence the enormous spread of inducers and their convergence onto a universal cancer phenotype; and as the re-profiling becomes more extensive so dynamic activities increase, which explains cancer's well-known progression.

Complication, Chaos and Complexity

The concept of Chaos is a mathematical description of dynamic states which are extremely sensitive, but not proportional to, initial conditions and do not follow straight lines. Extensive work by Aon and Cortassa [4,5] has described in detail how Chaos can be applied to cell systems and signalling, and explains for example generation of

Reproduced from reference [1] by kind permission of Elsevier Press.
Glycol=Glycolysis; E.R=Endoplasmic Reticulum
Upper: Cells exist as non-bounded microdomains (shaded motifs), each having multiple entry and exit points connected by fractal energy dissipation. They may be of quite different sizes with different phases, spatial organisations and multiple activities proceeding simultaneously. Environments are created under chaotic conditions by entropy determined by structure and enzymic activity etc.; red and green arrows indicate corridors of entropic flows. Cell function is a multi-input process determined by summation of thresholds from independent origins via fractal distributions.
Lower: Changes in initial entropic states e.g., from new proteins or constitutive activity migrating into crowded environments, re-organise microdomain connectivity, creating regions of repulsion and/or attraction. These are reflected in the entropy map and thus changed environments for threshold activations. In cancer, the map is re-configured to maximise energy dissipation through dynamic activities (motility, proliferation etc).

Figure 2: An alternative model based on entropy.

functional periodicities in neural networks and its involvement in how cells organise structure, involving thermodynamic entropy [5]. The term "entropy" is also applied to slightly different contexts; in tissue analysis for example it refers to degrees of deviation from a predicted/observed end-state [7] where energy reaches a minimum state. These publications are highly lucid descriptions of quite complicated mathematical concepts which are central to understanding how cells work. There is a linked mathematical discipline of similar extreme relevance, that of Complexity, related to Chaos [7,8]. In complex

systems new and unpredictable consequences arise (emergent properties) even if individual components are definable. Thus, while complicated systems may have many components and be understood (for example a car), in complex systems unpredictable events arise - in the car analogy motorway hold-ups associated with how drivers behave. Complexity is often encountered in business and population models, but it can address problems at the molecular level such as "molecular crowding", i.e., that reactions do not take place in empty space, but that presence of other molecules results in unforeseeable consequences. Molecular self-organisation/assembly is another where the products possess distinct properties unrelated to the components. Such concepts are clearly central to signalling. For example, that diffusion can account for the rapid transfer of molecules in signalling seems most unlikely.
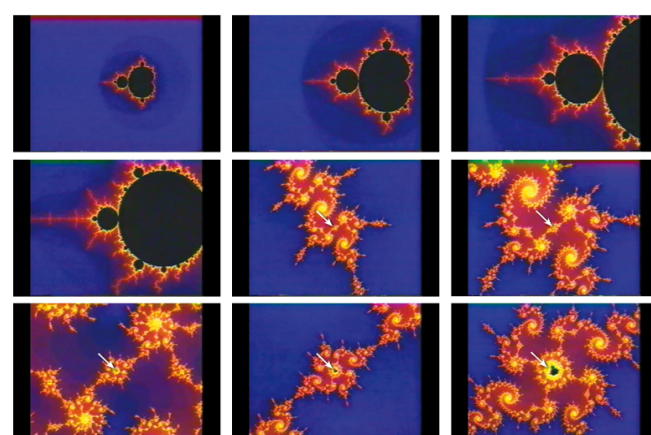
Reproduced from reference [1] by kind permission of Elsevier Press.
Glycol=Glycolysis; E.R=Endoplasmic Reticulum
Extracts from a video sequence in reference [1]. In the first frame top left the shape is seen to be bounded by an irregular circumference. As the shape enlarges, this circumference appears to be made of similar out-pouches. Each of these pouches then starts to replicate its own shape. Each new outpouching then generates further ones all linked by similar extensions and enlargements. This can be viewed as representing an entropic map. As energy is dissipated in an original focus, it radiates into a similar "shape". Molecules entrapped within one shape or possessing appropriate attributes can enter another when conditions are thermodynamically permissive and their interaction then creates an extension of the entropy map, for example, the white arrow. Because of the fractal organisation, widely different foci can function and many signalling systems can co-exist and meet, but also be integrated into the whole. The entire map is highly fluid. The links between foci can be thought of as entropic "corridors" between sites and the spaces within the shapes as nano-domains in Figure 2.

Figure 3: Fractal dynamic metamorphosis illustrated by Mandelbrot figures.
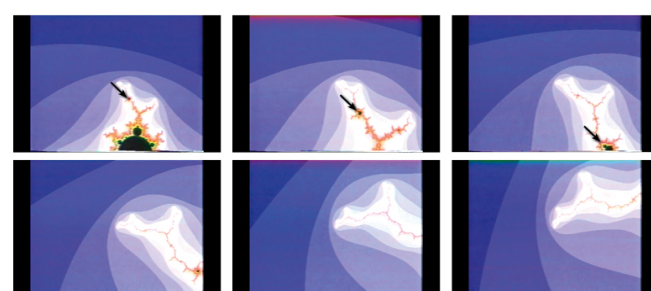
Reproduced from reference [1] by kind permission of Elsevier Press.
The fractal extension is deviated by its immediate environment represented by the blue outline. Black arrow identifies an emerging fractal.

Figure 4: Effect of an Attractor on fractal distribution.

While molecular motors actively transport molecules they also change cytoplasmic motion and influence cytoskeletal assembly [9]. A gain, formation of intracellular particles is dependent on cell size seemingly through alteration in cytoplasmic phase transitions [10]. Both illustrate the emergence of new properties. So, while signalling concepts are entirely valid for unravelling sequential events and most likely components, understanding of how they integrate and create emergent properties are not predictable, but are the essences of the signalling network.

Towards Re-thinking Networks

We know very little, for example, of how biochemical reactions are determined by molecular crowding, nor how such vast numbers of "pathways" can co-exist and be co-ordinated. That cells manage it is proof that solutions readily exist. Currently, this is being dissected using, for example, mathematical analysis of connexions/relationships between pathways [4] which has revealed a great deal. However, it is dependent on the data making those connexions which, as above, is becoming progressively more diffuse as more connexions and components even for established pathways are uncovered. While analysis of connexions can elucidate how systems can be put together sequentially, we know little about the *in situ* variables that decide actual responses within a network: concentrations of and equilibria between signalling components, effects of molecular crowding, formation of supra-enzyme-complexes and physical organisation, real-time measurement of how individual molecules move, precise energy balances etc. This is where biology, biochemistry and physics can work together using all the powerful tools available. For example, linking various alterations in metabolic pathways/networks to disease, e.g., cancer, is under close scrutiny. But arguably there are many ways of producing energy and what really matters is where and how it is used to drive a disease state expressing alternative signalling (this is discussed in [1]). Understanding this through co-operative projects may well create new pharmaceuticals targeting entropic states/locations driving whatever signalling process is changed e.g., in ageing and cancer.

Conclusions

Signalling has come a very long way. But, while much is known about components, there is a growing problem of ever-increasing data to put together. Part of the problem is that biological and molecular sciences are largely unaware of the comprehensive work being undertaken in other disciplines such as Systems Biology and Mathematical Physics using their results. Conversely, can the latter produce predictions on network behaviour that biologists/molecular science can explore? What do the Physical sciences need from biologists to enhance/test their detailed models; and what can physical sciences do to assist biologists understand their difficult areas where the mathematics is intense? One possible multidisciplinary area that appears to be unresearched is re-creating proposed signalling networks using purified, cloned components in progressively molecularly crowded conditions to elucidate principal driving mechanisms and behaviours during signal transduction in complex environments. We also need to know much more about single molecule distributions, and to quantify the components of proposed pathways, distributions, interaction equilibria etc., to feed into models. The time is ripe to rethink how signalling can possibly work using complex models reproducing "real" states but to do this requires that biological sciences and physical sciences are completely integrated into research programmes on cell signalling.

References

1. Garland J (2013) Energy management - a critical role in cancer induction? Crit Rev Oncol Hematol 88: 198-217.

2. Foffi G, Pastore A, Piazza F, Temussi PA (2013) Macromolecular crowding: chemistry and physics meet biology (Ascona, Switzerland, 10-14 June 2012). Phys Biol 10: 040301.

3. Floor S, van Staveren WC, Larsimont D, Dumont JE, Maenhaut C (2011) Cancer cells in epithelial-to-mesenchymal transition and tumor-propagating-cancer stem cells: distinct, overlapping or same populations. Oncogene 30: 4609-4621.

4. Aon M., Aon S., Lloyd D (2011) In: Meyers R (ed.) Chaos in Biochemistry and Physiology. Meyers Encycolopedia of Molecular Cell Biology and Molecular Medicine. Wiley-VCH Verlag GmbH & Co, USA.

5. Aon MA, Cortassa S, Lloyd D (2000) Chaotic dynamics and fractal space in biochemistry: simplicity underlies complexity. Cell Biol Int 24: 581-587.

6. Kayser K, Kayser G, Metze K (2007) The concept of structural entropy in tissue-based diagnosis. Anal Quant Cytol Histol 29: 296-308.

7. Coveney PV, Fowler PW (2005) Modelling biological complexity: a physical scientist's perspective. J R Soc Interface 2: 267-280.

8. Koutsogiannouli E, Papavassiliou AG, Papanikolaou NA (2013) Complexity in cancer biology: is systems biology the answer? Cancer Med 2: 164-177.

9. Brangwynne CP, Koenderink GH, MacKintosh FC, Weitz DA (2008) Cytoplasmic diffusion: molecular motors mix it up. J Cell Biol 183: 583-587.

10. Brangwynne CP (2013) Phase transitions and size scaling of membrane-less organelles. J Cell Biol 203: 875-881.