What do genetic screens tell us about the inner structure of biological systems in developmental and cell biology?

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NOTE: This is not a review (this is not the place for such things). This is a commentary, a couple of rough notes unpolished and free in style, an attempt to generate discussion and debate, of pouring out some thoughts. One important thing, in case you reach the end: I believe what I say here. It is likely that this will evolve. If you have any thoughts or comments, do not hesitate to contact me (ama11@hermes.cam.ac.uk). A PDF version of this can be downloaded from here.

"We are confronted by a nonlinear system the theory of which is fragmentary, complex and confused" (F. Crick talking in 2003)

A physicist's thought: difficult business Biology. ……..

Sometimes I wonder if we have not lost the plot or maybe, as we have been told a number of times, whether Biology is just '*different*'……*different* from, for example, Physics. I guess this comes from the fact that much of modern Science likes to be modelled on Physics and some of us just suffer from Physics envy. Well…..perhaps Biology is indeed **different**. The differences between physical and biological systems have been dissected in a few (not many) interesting pieces (Hartwell et al. 1999; Gunawardena, 2012; Roth 2011) and it is important to bear them in mind when thinking not only about what we want to know, but also about how we want to go about finding out what we want to know and, more important, what we should accept as an explanation. The second issue, the methodological one, is the main issue here.

What we want to know is how living systems work, how they are organized and structured. This for two reasons: to know and, also, to harness this knowledge to improve our quality of life. The leading light in this process of discovery has been, is, and will continue to be Genetics and there are good reasons for this. Through a combination of imagination and logic, the realization that phenotypic defects are associated with mutations in specific genes

and that one can use molecular biology to find out what those genes code for, and Biochemistry and Cell Biology to learn how their products work, has allowed us to build a logic and a formidable technical and intellectual arsenal that underpins our current image of living systems. In terms of the basic processes (metabolism, replication, transcription and translation) this picture sort of works, by which I mean that we can harness it for our interests; whether it is to make better (or different) wine or to engineer insulin. But at other levels, things are still a long way to prove useful and these 'things' are important to.

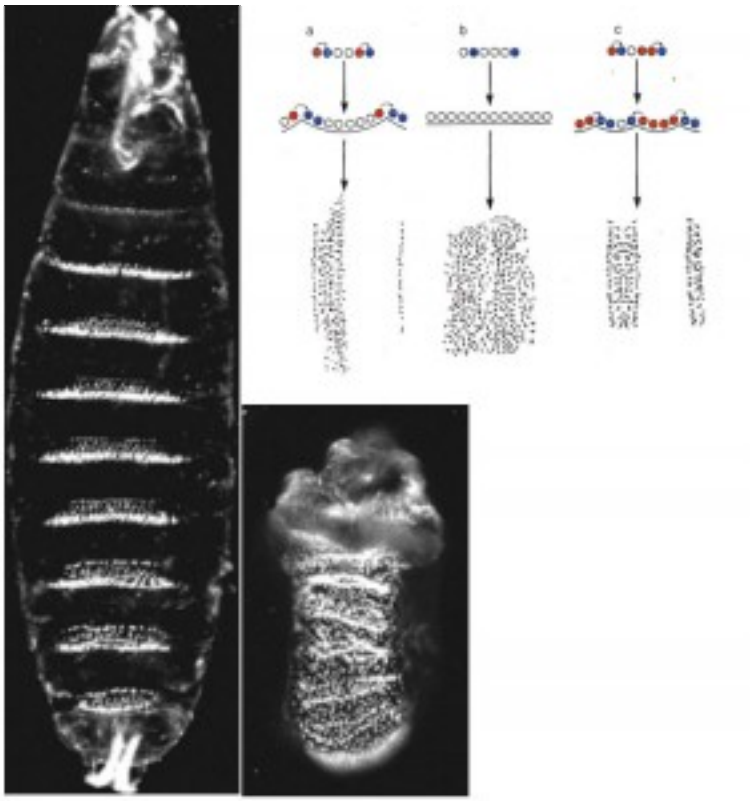
The tools of genetics (classical or molecular) are simple: mutation, phenotypic analysis, epistases and interpretation and synthesis of the results. At the heart of these there are 'screens' and it is the nature of screens and their value in today's science that I want to address here. Screens have become extremely prevalent in biological research but I believe that nowadays they are double-edged swords. If properly conceived and set, they can (they might) tell us something useful about the process of interest. However, nowadays, most of the time they return two answers: either more components of the cell which we cannot place into any framework, or the response of the system to a complicated situation, an adaptive response. Biological systems are, by their nature, reactive i.e. they will always respond to selection and therefore a genetic screen, which by nature is a selective process, will always work i.e. produce, as it should, mutants but….will it teach us something new, interesting, useful or….will it just say that the system responds to our treatment?

The origin and meaning of word 'screen' and of the notion of a 'genetic screen'

Let us go back one step. What is a genetic screen? The term 'screen' is derived from the acception of a **screen** (according to www.thefreedictionary.com: *A coarse sieve used for sifting out fine particles, as of sand, gravel, or coal*) which then, of course, gives rise to the term **to screen** (*same source: To separate or sift out (fine particles of sand, for example) by means of a sieve or screen*). So, the sieve or screen creates a criterion to select for something wanted. Humans have been unconsciously screening genetically for thousands of years, and farmers in particular have been doing this consciously for a long time, as this is how we have got our crops and domestic animals. In many ways, the initial studies of heredity were very closely linked to this type of screen and it is probably not surprising that Mendel was working with crops of the kinds that farmers played with. Nevertheless it was probably TH Morgan who set out to do a genetic screen consciously, when he began to grow *Drosophila* looking for spontaneous large phenotypic changes, mutations (Allen 1978). He

spent over two years looking for some discontinuous change in the outer appearance of the fly, without much joy; collecting small variations until he found *white*. The rest, as they say, is History and*Drosophila* became the darling of the mutant hunts, or screens, for many years. Mouse genetics, though with a recent history anchored in tumour biology (Paigen, 2003) also has its roots in breeders of their phenotypic aspects: the fancy mice breeders in the Orient which provided a basis for the research strains that would become popular later (http://research.jax.org/mousegenetics/development/history.html and http://www.hhmi.org/genesweshare/d110.html). The discovery that radiation and chemicals could induce mutations, changed the way mutants were isolated and the rate at which they appeared and were collected increased (Carlson 2004).

The ability to induce mutations changed the game and the notion of a screen; a directed selection of certain kind of mutants, began to take shape. Phage, *E. coli*and yeast benefitted very much from this and in this manner we learnt about the metabolic pathways, the genetic code and the basic processes of information transfer in living systems. This knowledge was gathered through mutant screens, which targeted particular processes and used mutants and double mutants to unravel them. These screens were very successful because the questions they asked (a screen always asks a question to the organism) were very targeted and the design was such that one always asked for rare events whose occurrence would be determinant and informative of the process in question. Thus, in these organisms one could screen $>10^6$ individuals, which is key on these rare events. This work created the foundation of Molecular Biology (read “The eigth day of creation” by HF Judson for a great account). In parallel, genetics had been applied, albeit in a small scale, to more complex processes, in particular the cell and developmental biology of organisms. However, the lack of clear questions and the difficulty of devising screens other than with low numbers of individuals, kept this work as anecdotal stamp collecting (see e.g Hadorn, 1961). Nevertheless, it created a foundation for what was to come. In particular it provided a flavour for the kinds of mutants which could be found and, more significantly, for the fact that one could deal with SOME of these processes genetically. Classic mutants like*bithorax* and *Krüppel* in *Drosophila*, or *T/Bra* in the mouse, are a product of this period.



Wild type Drosophila larva (left) and mutant for wingless. From the Tübingen screens. On top how these mutants helped us understand the cellular basis of the cuticle pattern.

S. Brenner had been working with *C. elegans* along the lines that genetics would provide the answer to your favourite problem, whether it was how the nervous system was built or how it works but it was in the early 1980s that C. Nüsslein Volhard, E. Wieschaus and G. Jürgens carried out a seminal screen in *Drosophila*for embryonic lethal mutations. This work not only transformed developmental biology but laid down the logistics of these experiments and showed their potential as a deep tool of discovery (Nüsslein Volhard and Wieschaus, 1980; Nüsslein Volhard et al. 1984; Wieschaus et al, 1984; Jürgens et al. 1984). Screens had been done before, but except on very rare occasions, looking for visible viable phenotypes and, in the few cases of lethals, in much smaller scale. This was a large scale experiment (no free lunch here for anyone), organized with an almost military precision which yielded an embarrassment of riches that kept at least two generations of graduate students and postdocs working on its product. The screen was successful because the mutants it yielded were informative about biological processes. It was a high level screen; it selected lethal mutations and then looked at the phenotypes. Nothing more, nothing less. One of the most significant findings was that the mutations could be organized in classes which meant that there was a logic to the processes mediated by the gene products (Nüsslein-Volhard and

Wieschaus, 1980). Of course, this had been seen before in bacterial research but here it was in relation to the making of an organism. The next few years confirmed this and, together with the molecular biology and related screens in *C. elegans* (see e.g Ferguson et al. 1987, Ferguson and Horvitz 1985) revealed that the components of the system and their function were evolutionarily conserved. This type of screen was extended and repeated to maternal loci and also to other organisms, in particular the zebra fish by C. Nüsslein Volhard herself with a large group of people, and much like in *Drosophila*, this screen (published in a special issue of Development in 1996) laid down the foundation of a field and generated work.

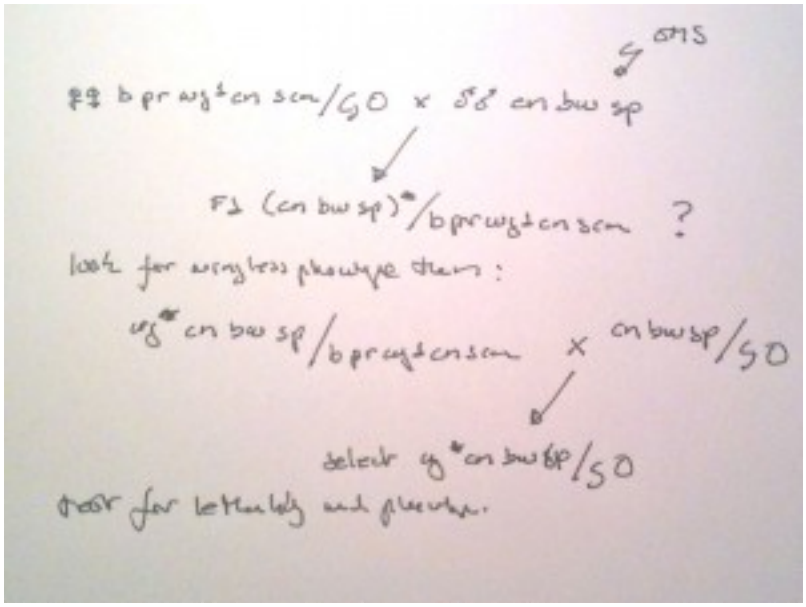
This is a good place to take stock. These screens worked well and the reason is that they were generic, that they looked at a process from a high level, that they asked very simple questions of how a process, a very general process as is the development of an organism, can go wrong. They provided information where there was ignorance.

Classes of screens

Broadly speaking there are three kinds of genetic screens: search for the effects of loss of function mutations and either suppressors or enhancers of other mutations. It is possible to present each of these in a variety of colours and flavours, which form the basis of the screen kit. The first kind (straight loss of function) is the one that was used in *Drosophila*, *C elegans and A. thaliana* in the 70s and the 80s and later zebra fish in the 80s and 90s, and requires large, but not impossibly large, numbers of individuals and a good logistics. Enhancers and suppressor screens are different. They start with a phenotype associated with a mutation and look for ways of either enhancing it (making it worst) or suppressing it (making it better). The little book “pushing flies” by Ralph Greenspan, explains much of this in simple and useful detail.

It should not come as a surprise that the first kind is easier than the second and that for enhancers screens to be useful, they have to be carefully thought out with a high degree of stringency. A very good example of an enhancer screen was carried out for *sevenless* in *Drosophila*, which yielded key elements of the Ras signalling pathway, thus revealing its linearity (Simon 1991); in this work *sevenless*was known to encode an RTK and using a temperature sensitive allele, the temperature of the incubators was exquisitely carefully set so that function was suboptimal but allowed for defects to be created by additional mutations in other proteins that interacted with Sevenless and decreased its function. Suppressor screens are more difficult to enact, but tend to yield riches, as one can be very stringent on

the question (the screen) such that the answer, rare as it might be, would be informative (interpretation being an important element here).



Outline of a screen for alleles of wingless

As the events in suppressor screens are rare, they require large numbers (usually $>10^6$ or 10^7) which can only be afforded in prokaryotes, yeasts, some plants or, of the model organisms, only *C. elegans* e.g Sundaram and Greenwald (1993) –though they have also been performed in *Drosophila* (Karim et al. (1996) and Ferguson and Anderson (1992)) with good results i.e. they produced informative outcomes. Still, because the nature of mutations is stochastic and the events are independent, the numbers to be screened do not determine the actual success but rather its probability. Nonetheless, the crucial things of suppressor/enhancer screens are the numbers, their thresholds and, in the case of enhancers, a stringent second screen.

As genetic engineering has created new tools for the controlled expression of genes as well as different manners to follow expression at the level of gene or proteins, the possibilities of screens have increased. Nowadays the repertoire is often limited, only, by the imagination of the experimenter. It is also possible to screen whole genomes and with the advent of libraries of siRNAs or shRNAs and controlled overexpression, it is possible to generate screens of gain and loss of function, of suppressors or enhancers, at will. More over, one does not need to rely on the organism to provide the phenotype as it is possible to engineer it by labelling

organs, cells and/or proteins with tags, fluorescent or otherwise. And this is the tip of an iceberg. The possibilities are enormous and rising. The combination of these technologies with improved tissue culture methods have allowed to move into mammalian territories and to explore mammalian genomes in different cell lines, most significantly, in Embryonic Stem cells (see for example Guo et al. 2011 and Yang et al. 2012)

All in all, genetic screens have become the corner stone of all biological research and, more than metaphorically, the daily bread of the biologists ……..and counting.

The good, the bad of genetic screens……. What do screens really teach us?

Genetic screens were celebrated in a special collection of Nature Reviews Genetics between 2001 and 2003 (the art and design of genetic screens:http://www.nature.com/nrg/focus/screens/index.html). They are used world wide and taught in undergraduate and graduate courses as the way to gain knowledge in biological systems. There is a good justification for this in the successes of the past (see references above). However, like everything that is powerful (and genetic screens are powerful), all depends on how it is used; the topical gun in the hands of a child comes to mind. In the case of genetic screens, they work, they produce mutants and thereby information which needs interpretation. However, as we have been advancing in our knowledge and technical prowess, I surmise that we have not changed our ways, we continue to harvest mutants and, perhaps, it is a moment to ask questions: what do we get from genetic screens now? More to the point, do they make a difference, a significant difference, to our knowledge? I would argue that for the most part, they don't any more, that at the moment we lack the framework and the knowledge to understand what they throw at us and that therefore in most cases, they provide elements of a curious collection of objects. Not always, not every screen, but most. Screens have become an easy road to a publication and all that this carries with it. Journals keep on publishing screens few of which are insightful. For the most part they end up, in the best of cases, doing little more than adding nodes to ever growing networks or providing new elements to processes for which we still do not have appropriate frameworks; rarely, do they yield something useful.

A good example of the good and the bad can be found comparing two published genetic screens in *Drosophila* geared to find the same things: genes that work with Notch (a classic gene encoding a single transmembrane receptor with important roles in development and disease). One of the manuscripts is sloppy, without a proper structure leading "to identify

nuclear import pathways and the COP9 signallosome as Notch regulators" and concluding that "that complex developmental processes can be analysed on a genome-wide level" (Mummery-Widmer et al. 2009). Perhaps it will not surprise you that this was published in Nature and was hailed as an important achievement and a good piece of work; difficult to see the reason for this or the insight of the work. On the other hand there is a much more laboured and controlled screen targeting the same problem, which "revealed several modules of unexpected Notch regulatory activity. In particular, we note an intriguing relationship to pyruvate metabolism, which may be relevant to cancer". Believe me, this one is good. It is sound, you get much of what you expect and more and all with proper controls (Sai et al. 2010). Whereas I would not put my money in the reproducibility of the first one I would happily bet for most of this one. It was published in Dev. Cell, though I know from the senior author, that this was not without aggravation and discussion (why do we have to always do this to publish in these journals?). The second screen is in vein of the 800,000+ flies of Karim et al (1996) with a similar slant, and is an example for whoever wants to undertake these experiments. But it is hard work, physical and intellectual.

Despite all the hard work, screens are about experimental design, organization and questions. There is no more to it. Most people can do them and, as I have said above, they pay: they do give mutants. The tragedy is that they are meant to help researchers and I bet you that many of them don't. For the most part one ends up with situations like that of the Mummery-Widmer paper, many mutants but….how do we know if they are what we want.

The problem lies not so much in the screen itself, as in the need to think carefully about the question one asks beforehand, in the need of a good framework to evaluate the results, and in whether looking for elements of a complex system (and let us not fool ourselves, this is all screens will give you), whether this is the way to go about it. These days, genetic screens are used to probe into the workings of highly connected protein networks but rather than taking this into consideration, the designs consider linear relationships between the existing elements which they confirm with a crafty use of epistasis (see http://amapress.gen.cam.ac.uk/?p=914). The terms upstream and downstream are often used in these contexts and this regard. However, highly interconnected non linear molecular devices are difficult to break and much less when they are thought to be linear in the design. What sometimes happens is that, inadvertently, one does a screen in a particular cell type in which some of the feedbacks are disabled or the elements do not work well and then one gets an answer. There are too many tissue and sometimes species specific connections

that can be misleading and the screen might reveal one of these in terms of a new element.

But the two biggest problems with screens today have to do with our lack of understanding of what I would call the ***'inner structure of biological systems"*** and how this affects the work.

The first one is that we do not look at what we should. At the helm of the cell is a set of proteins which we already know but whose workings (in the system sense) we do not understand. I am referring to systems like cell adhesion, traffic or signal transduction. The reason for a screen is often, always, to find a 'new' gene to then undertake a salesmanship job (if you think I am being cynical, think again on what you know). As indicated above, the best way to do this is with suppressor screens but even these sometimes might not work, so in most cases one sets up conventional loss of function screens that tend to be somewhat baroque. What happens then is a familiar story, half of which never sees the light of day. The one thing that most screens will do is to yield, in addition to mutants we have not seen before, mutations in genes and systems we already know; in particular in elements of Wnt and Ras signalling, cell adhesion and chromatin remodelling systems at the top of the list (and quickly behind elements of traffic and chromatin remodelling proteins). As this is not what we are looking for, we throw them away…….at our peril I would claim….and proceed to try to fix the 'new' things we have obtained and to connect them to some of the things/genes we already know. Genetics is sufficiently flexible to detect interactions which are usually good enough for an argument of relationship. We find our 'pathway' and presto, we can write a paper. As the point of the screen is to find new elements, so the point needs to be the 'new' things. The result is that we are left with a 'new member' of this or that pathway or a protein of unknown function which we hastily will find a way of linking to something we know. I am sure that if, instead of looking at the bright new things, we looked at what we know and asked why do we get this and not that, why X appears with Y in this screen and with Z in that other one? Why do we get this type of allele here and in some other screen we don't? I bet you that if we were allowed to ask these questions, we would learn much more about the system than we do from the new genes which tend to be most of the time like characters in search of a plot.

The second reason why screens are problematic is that biological systems are highly redundant (in the sense of having multiple backups) *and have evolved to respond genetically speaking, to selective pressures and a screen is, basically, a selective pressure.* A biological system will always respond to the call of a screen by giving you mutants, even when we place the system in extreme situations. Take some of the screens in *S. cerevisiae* which, for example, making use of auxotrophic selection on Histidine, one can ask cells which lack

crucial deletions of the HIS3 gene regulatory region which abolish the expression of the gene, to express the gene (Oettinger and Struhl, 1985). They do and (some of) the mutations turn out to be informative about the mechanism of transcription. This is dramatic, like resurrecting a dead individual. Similar experiments have been done in *E. coli*, where the classical work of Barry Hall (Hall and Hartl, 1974; Warren, 1972) in which he asked *E. coli* lacking the ß-galactosidase gene to grow on lactose, set up a paradigm one should think about when doing genetic screens. In the experiment he found the mutagenic activation of another locus, call *ebg*, which can do the job of LacZ.

In many ways, either by mutation and selection or simply by selection, when challenged with a screen, the system will respond and will give you mutants. These are very extreme cases so it should not be a surprise that less stringent circumstances will yield more and more varied mutants. When a biological system is asked to give mutants, it does. In many ways that is what has been designed to do so as to respond to evolutionary pressures. *Screens are doing only, what natural selection does: select and this will always happen.*

So, one has to be very careful that when one sets a genetic screen one is asking a question about the process of interest and not asking the system how it adapts. We know that biological systems can adapt and no better example of this than what has been uncovered during the widespread use of siRNAs, RNAis and shRNAs: the immediate phenotype induced by these methods can be stronger and sometimes different than that of the genetic mutant. The reason is likely to have an element of the fact that the genetic mutant adapts and therefore, after a few generations, the organism (or the cell) has changed its phenotype (it needs to do this to survive). There is no clear proof of this, but it is all over the place and people have used this in their work (see Martello et al. 2011 for a good awareness of this). The degree of adaptation can be rather surprising and if you want an extreme case look at the work of Coudreuse and Nurse (2010) in which they can eliminate the mayor regulators of cell cycle progression in S. pombe and still achieve regulation through controlled expression of an engineered cycline dependent protein kinase (an imposed adaptation).

So, beware of asking questions about adaptation. One needs to learn not to ask a question about how the system can adapt to a situation but rather, how the system works. I would argue that the more we focus our questions, the more we are moving away from how the system works. And remember that Genetics will always, only, give you parts.

…and the ugly (or a swan in disguise?): The inner structure of biological systems

And thus to the end with what I would call “the inner structure of biological systems’, which is what consciously or (mostly) unconsciously we are probing in the genetic screens. It is early days, we are just beginning to get glimpses of this but the feeling one gets (and here I should say “I get”) is that the essence of this structure that we call a cell (and by extension a multicellular organism) is a collection of small networks, in the ‘alonesque’ (for Uri Alon) sense, each of which performs a logical operation (Alon 2007). These small networks are loosely connected amidst them (have discussed an early version of this in Trott et al. 2012) and the connections, which are really biochemical, fluctuate. There might be devices, molecular devices, whose function is to strengthen these connections in a cell type or situation specific manner, and I believe that Wnt signalling can be interpreted in this manner (Martinez Arias and Hayward 2006). These connections create an operational hierarchy with a lot of basic (in terms of the needs for the cell) but not regulatory stuff in the periphery, highly connected to the core (see below). The important aspect of such a structure is that it is reactive, that it can reorganize itself in response to its environment, and that it can adapt.

The early screens were unaware of this (if it exists). Unconsciously they targeted the key components of these networks, the hubs, and found that when mutating them many processes went wrong. This is what you expect from such screens because they look at and thereby select for those phenotypes. Having obtained the hubs and some of the networks as a result of these hits, we set up schemes to target the system in more fine manner and by doing this we turn our attention to the redundant networks in the periphery (without knowing and without knowing how they look which means that, often, we fail to understand). The new crop of screens is not very precise in terms of looking at the structure of the system, they are sophisticated but often all they ask is how the system rearranges itself in a stressful situation. The screens that work (i.e that provide information about the system) are those that target the structure and function of this core-networks, we could call them ur-networks.

Screens are expensive and time consuming and it is for this reason that we should think why do them and what we expect from them. Then target exactly that rather than embark in fishing expeditions for new genes, which will only add elements for a confusion. Most importantly, at this point in time before we collect more components we need a framework, or frameworks, of how the cell, its sensory system, that Dennis Bray would say, is organized and responds. We have to separate (and this is difficult) the actual working of the system from its adaptive nature…..but maybe this is not possible. But we need to try. One suspects

that the outcomes of the screens are telling us something about the inner structure of the cell and we need to figure it out if we are going to make progress in our understanding of the function of biological systems. The ideas of Dennis Bray, clearly outlined in his book Wetware, are a very good reference in this undertaking.

An outline of what I mean by the 'inner structure of biological systems' will be published here shortly in the form of an Appendix to this commentary.

In summary

An understanding of the structure of the cell, physical and functional, will lead to new findings and the rational design of experiments, in particular screens. Most urgently, we need to figure out efficient ways to target connectivity in genetic screens rather than components, which is what is being done at the moment.

Epilogue

A biologist's thought: Biology is indeed difficult……but rewarding when you understand something…..or you think you do.

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