THINKING DEEPLY

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Prevailing scientific views guide research. However, as the old saying goes, "not all that glitters is gold", that is, "wide acceptance" does not necessarily mean "on the right track". Following a common but incorrect view, may lead to misleading results. Anyhow, as the right track is not "signposted", to find it may require deep thinking. This means being open to the results, rather than fit them to preconceived ideas. By exploring where new results might lead, you will find other ways of thinking. By looking at data from different perspectives, you will be able to construct a bigger picture. Deep thinking gives you freedom of thought with which to choose the path to follow, based on your own criteria. Importantly, deep thinking should not be considered a theoretical endeavor with little impact at the experimental level, but rather part of the daily life of a researcher. Here, I will discuss four common views encountered during one year of research.

The germ layers form during gastrulation

After many divisions, the zygote forms a solid sphere of cells called the morula. Later, the morula becomes hollow, giving rise to the blastula. The latter is a single-layered structure. During gastrulation, a series of cell movements transform the blastula into a multilayered structure, the gastrula. These germ layers give place to the different tissues and organs of the body. It is commonly thought that the formation of the three germs layers of the vertebrate embryo (i.e., ectoderm, mesoderm, and endoderm) is complete by the end of gastrulation. This means that the gastrula would already "contain" the whole body. However, it has been shown that, at least in some vertebrate species, gastrulation does not give rise to the whole body, but to the anterior part only (reviewed by Steventon and Martinez, 2017). The formation of the germ layers continues after gastrulation, by a mechanism that involves different molecular signals (i.e., molecules that trigger a cell response; Goto, et al., 2017). This mechanism elongates the body, thereby forming its posterior part. In other words, gas-

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trulation is the most important day of your life, as Wolpert said, for half of your body; for the other half is body elongation.

This has a direct implication at the experimental level. As ethical reasons preclude embryological studies in humans, researchers have focused on reproducing organs in a dish. The idea is to transform an aggregate of pluripotent cells (i.e., those capable of differentiating in any cell type) into an organoid—a 3D structure that mimics an organ (e.g., Lancaster and Knoblich, 2014). Similar to the case *in vivo*, the first step to generate an organoid is to induce the formation of the respective germ layer, i.e., the layer that gives rise to the organ. How this is achieved will depend on which of the previous views is held. Under the prevailing view, the signals involved in gastrulation are added to the culture first. The ones involved in body elongation are seen as signals that cells receive *after* gastrulation, and therefore, they are added second. According to the alternative view, which some authors have traced back to the nineteenth century (Henrique, et al., 2015), there is not one, but two routes of germ layer formation. Confusing these two routes, like in the above protocol, may lead to misleading results. However, established protocols to develop some tissues *in vitro* are based on the prevailing view.

Transcription factors are located in the nucleus

Transcription factors are proteins that regulate cell response by binding to specific regions of DNA, thereby turning "on" and "off" target genes. Due to their role, it is commonly assumed they should be located in the cell nucleus, where most of the DNA is located. If a transcription factor is found outside the nucleus, it is generally thought that the detection method did not work and that the data should be discarded. However, this view is not strictly correct. Transcription factors can be found at different subcellular locations, but it would only be when they are in the nucleus that they *directly* turn "on/off" their target genes. Indeed, transcription factors, like most proteins, are synthetized in the cytoplasm, from where they move to the nucleus. Taking into account the complexity of the regulatory mechanisms, it is probably as informative to find a transcription factor outside the nucleus as it would be to find it inside the nucleus (e.g., Hader, et al., 2010). Thus, to follow such preconceived idea may result in relevant aspects of a process being overlooked.

All pluripotent stem cells are the same

Although pluripotent cell lines are from different origins, it is commonly thought that they are all equally pluripotent, that is, they can differentiate into any cell type with the same efficiency. An increasing number of studies are showing that pluripotent cells are not all the same, but that significant differences exist in the efficiency with which they transform

into different tissues (e.g., Tewary, et al., 2019). This means that it would no longer be appropriate to use any cell line to derive any organoid, as is commonly done. Depending on the organoid to be generated, some cells lines would be more appropriate than others. For example, to generate an organoid of mesodermal origin, it would be necessary to choose a cell line capable of differentiating into mesoderm with high efficiency, and so on. When using cell lines with different efficiencies, it would be necessary to optimize the protocol for each of them. Furthermore, as cell lines may change after a long term in culture, it would be recommendable to check their efficiency with some periodicity. Taking into account the relevance of this point, testing the suitability of a pluripotent cell line for a specific aim will likely be a routine step in future works.

Living matter is passive and non-intrinsically ordered

When single pluripotent cells are cultured in suspension, they form cell aggregates. The general idea is that cells sink to the bottom and, without interacting with each other start to divide, forming an aggregate. Time-lapse imaging of these cultures shows that cell aggregation is more complex (personal observation). Depending on the cell density, aggregates do not form only by cell division, but also by cell clustering, i.e., cells close to each other come together to form an aggregate. At a certain cell density, the latter is the only aggregation method. This means that the aggregates are not necessarily monoclonal as is sometimes assumed. Most importantly, cells do not come together by a passive Brownian motion, but rather move actively and directionally: they "crawl" on the bottom of the dish and move towards a nearby cell or cluster. Some aggregates also grow by actively moving around and engulfing surrounding cells or aggregates. Furthermore, cells not only aggregate, but also disaggregate and re-aggregate. According to these observations, cell aggregation does not appear to be a passive process, in which cells just divide, but a dynamic process resulting from cell interactions, i.e., a self-organizing process. In support of this hypothesis, these cell aggregates organize into complex structures called cysts, without the addition of any molecular signal (an external input) to the medium, i.e., they actively shape themselves (Taniguchi, et al., 2015). How cell aggregation occurs would probably not affect the way organoids are cultured, however, the nature of living matter continues to be a long-standing controversy with far reaching implications (Linde-Medina, 2010).

As can be seen from these examples, to question prevailing ideas have disadvantages. Rethinking a protocol would require more effort than just following it. Considering that pluripotent stem cells are not equal, means having to develop more than a single protocol. Considering that

transcription factors can be at different locations may invalidate interpretations based only on quantitative data (e.g,. qPCR data). Under the publish-or-perish dictum, these considerations may put more pressure on you. When moving away from commonly accepted thoughts, your work will probably attract more critics than supporters, with the difficulties this may entail. But if deep thinking does not assure success, the question is: who cares? Those that believe in better science.

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