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In a century from agitated cells to human organoids

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ABSTRACT

Reaching back more than a century, suspension cultures have provided major insights into processes of histogenesis; e.g., cell communication, distinction of self/nonself, cell sorting and cell adhesion. Besides studies on lower animals, the vertebrate retina served as excellent reaggregate model to analyze 3D reconstruction of a complex neural laminar tissue. Methodologically, keeping cells under suspension is essential to achieve tissue organisation *in vitro;* thereby, the environmental conditions direct the emergent histotypic particulars. Recent progress in regenerative medicine is based to a large extent on human induced pluripotent stem cells (hiPSCs), which are cultured under suspension. Following their genetically directed differentiation into various histologic 3D structures, organoids provide excellent multipurpose *in vitro* assay models, as well as tissues for repair transplantations. Historically, a nearly fully laminated retinal spheroid from avian embryos was achieved already in 1984, foreshadowing the potential of culturing stem cells under suspension for tissue reconstruction purposes.

1. Cell culturing reaches back into the 19th century

In the nineteenth century, the cell theory had been shaped by Schleiden, Schwann (1839), Virchow ("*omnis cellula e cellula*") and others (see Jahn, 2002; Gilbert and Barresi, 2020). With his 1907 experiment keeping frog neuroblasts in a lymph-containing medium, Ross Harrison is considered father of cell culture (Harrison, 1907). However, already towards the end of the 19th century, embryologists investigating fertilization and cleavage processes had paved the way into cell culturing. Beginning with Wilhelm Roux́s experiments on cleavage of frog embryos (Roux, 1885), more culturing experiments followed with macerated plants and lower animals, like sponges or sea urchins. Remarkably and mostly forgotten, such experiments were performed in agitated glass dishes including salty sea water, whereby the dispersed cells were kept under suspension. Thus notably, cell culture did begin with suspension cultures, also called rotation or shaking cultures. Only later other types of cultures were introduced, i.e., hanging drop (as Harrison did, above), or standing cultures, the latter of which later-on became the gold standard of cell culturing. In tracing back the origins of cell spheroids and organoids, remarkable reaggregate research of Henry van Peters Wilson and the eminent Hans Driesch deserve particular merits (Fig. 1, left).

2. Regulation and regeneration - Drieschś "Schüttelversuche" on sea urchins

In an attempt to support the then standing *mosaic theory* of Wilhelm Roux (1885) and the *germ plasma theory* of August Weismann (1892; see Jahn, 2002), Hans Driesch investigated developmental potencies of cleavage cells of sea urchin embryos (Driesch 1892, 1893; see Jahn, 2002; Gilbert and Barresi, 2020). One type of experiment achieved high relevance for all biology, since it brought the topics of *regeneration* and *regulation* into the focus of biologists. These experiments were called „Schüttelversuche" (engl., shaking experiments), since dispersed cells from 4 to 8 cell stage embryos were kept under suspension. When Driesch removed the fertilization envelope from a 4-cell embryo and isolated the four cells, then each cell could develop into a smaller, but normal pluteus (i.e., larva of sea urchins). What Driesch had discovered was the capacity of - what became called „*regulation*" - that each of these four cell possesses the capacity to balance out for what is missing in absence of the other three cells. In other words, each cell at this early embryonic stage is able to form a complete animal: in fact, this fully complies with the definition of a *totipotent* embryonic stem cell (ESC). Thus, Driesch should be considered the father of stem cell biology. Indeed, Drieschś findings by his simple *in vitro* experiment (which

Abbreviations: Ac, amacrine cell; Inl, onl, cell layers corresponding to inner and outer cell layer; PESC, pluripotent embryonic stem cell; Ipl, opl, synaptic layers corresponding to inner, outer plexiform layer; OS, outer segments of photoreceptors; RPE, retinal pigmented epithelium; Ros, PR rosette; RPCs, retinal precursor cells; Vis, visinin; Vim, vimentin; SAC1, 2, starburst amacrine cells of type 1, 2.

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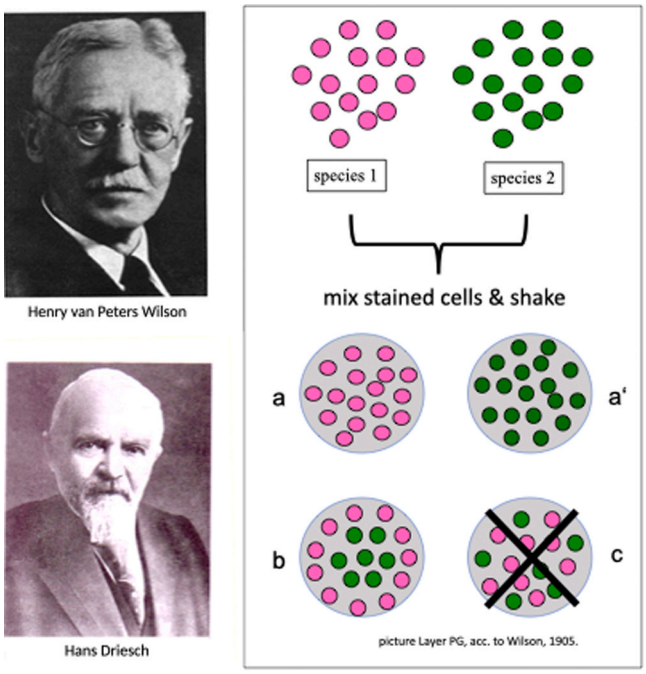

Fig. 1. (on left) At the turn into the 20th century, discoveries of two eminent embryologists - Henry van Peters Wilson (upper) and Hans Driesch (lower) - provided basic insights into phenomena of cell reaggregation, communication and their regeneration into whole animals. Frame (on right) sketches Wilsońs mixing experiment using dispersed sponges. Two sponge species were differently stained; their dispersed cells were mixed *in vitro* and reaggregated under suspension (upper). Results revealed that (a, a') either both types of cells were completely segregated within individual aggregates; or (b) different cells were segregated within the same aggregate; aggregates missing any spatial order were not found (c). Note that in a-c individual cell reaggregates are represented, not dishes. Further see text (e.g., for Drieschś experiments).

manually was difficult!) was a milestone influencing developmental biology (i.e., topics of cell determination, regulation, regeneration, induction, tissue interactions, nature vs. nurture, etc.) throughout the last century until today. Driesch was startled by his findings, yet interpreted them aptly by his famous sayings „the prospective potencies of cells are greater than their prospective fates“, i.e., during normal development not the full capacity of each cell is actually being exploited. Finding no materialistic explanation, he suggested that each cell must be driven by a transcendent „*Lebenskraft*“ („force for living“), thus engaging in *Neovitalism* (see Layer, 2022).

3. Reaggregation of dissociated sponges reveal cell communication and “sorting-out” phenomenon

Another experiment, published by Henry van Peters Wilson in 1907, again provided insights into basic properties of cells. When dispersed cells from sponges were kept agitated in sea water, cells readily would reaggregate to form small cell clumps, indicating their tendency to stick to each other. Eventually, whole sponges would regenerate from these cell clumps. Then cells from two closely related sponge species (differently stained) were mixed and agitated in one glass dish (Fig. 1, right). Again, cells would readily aggregate and form coherent clumps, whereby two scenarios could be distinguished: i) stained cells from the two species were completely separated within different cell aggregates (upper), or ii) cells were locally segregated within one aggregate (b); but remarkably, within one aggregate they were never distributed by chance (c). Indeed, these results from a simple series of reaggregate experiments were as stunning as they were revealing: cells not only adhere to each other, but they prefer sticking to similar cells; they have a tendency to stay together, avoiding contacts with (more) foreign cells: it was said that cells „sort out“ (cell-sorting phenomenon). In fact, experiments like this around the turn of the century revealed basic cell properties: it was nothing less than the discovery of cell communication, of self-nonself recognition and sorting-out, which only from the 1970ies onwards would become intelligible at a molecular level (cell adhesion molecules, membrane constitution, cell signaling, membrane receptors, etc.).

4. Reaggregate approaches modelling animal regeneration

Following Wilsońs telling discoveries, more experiments on reaggregation of dispersed (or at least disrupted) whole animals in a culture dish ensued. Using different sponge species (*Porifera*), De Morgan and Drew have repeated Wilsońs experiments and could confirm most aspects of his results (De Morgan, Drew, 1913–1915). Galtsoff worked with the sponges *Microciona* and *Cliona* in suspension culture, detecting that dispersed cells were moving around like amoebae (Galtsoff, 1923). It was also during the first half of the 20th century that the topic of biologic regeneration gained much attention. For instance, cnidaria and planaria (flat worms) can be cut into pieces and will regenerate whole animals. While hundreds of flat worms can be regenerated from one animal, earth worms (*Lumbricidae*) when cut into two pieces, only one of them will regenerate into a complete worm. Then, it was Alfred Gierer who introduced the sweet water polyp Hydra as a study model for biologic pattern formation and regeneration. After cutting Hydra into pieces and culturing dispersed cells under suspension, complete living animals could be reconstituted (Gierer et al., 1972). In the wake of this founding *polyp school* at the MPI in Tübingen/Germany, several generations of researchers established Cnidarians (Hydra, Nematostella) as most revealing models of developmental genetics and regeneration (Gierer, 2012; Boehm et al., 2013; Holstein, 2023).

During the first half of the 20th century, the topic of regeneration and regulation achieved high attention. Some fishes and amphibia can regenerate whole body parts. For instance, Urodeles can regenerate several parts of their body after their loss (newts, tail of Salamander, etc.), while Anura (frogs) have much more restricted (mostly to embryonic/larval periods) capacities to regenerate their tissues. How can a lizard easily regrow its lost tail? Retinas in fish and also amphibia can regenerate (at least up to some stage): how is all this possible, what are the mechanisms of regeneration, what could explain the distinct species differences? In all vertebrates, blood cells, skin, gut epithelia and liver continuously regenerate (or better, self-renew). Thus, suspension cultures from separate tissues or organs were initiated in the 1940ies and contributed much to the topics of physical maintenance and self-repair.

5. Avian retina as reaggregate model

Being interested in the phenomenon of biologic regeneration, Aaron Moscona became one of the fathers of the technique of cell reaggregation and of transplantation of reaggregated tissues back to the embryo. Working with dissociated cells of various origins, e.g., kidney and lung, he introduced the embryonic chick retina as a suitable model for reaggregation experiments. Including in his retinal cultures black cells from the pigmented epithelium (RPE, surrounding the retina) was advantageous, since cell markers were not needed (Moscona, 1959). Similarly, Japanese research on *in vitro* tissue regeneration was outstanding; e.g., providing insights into the reaggregation process of retinal cells (Fujisawa, 1973).

Applying also the chick retina reaggregation approach, Malcolm Steinberg became well-known for his so-called „differential cell adhesion hypothesis“ (DAH; Steinberg, 1962, 2007), which was an attempt to explain the phenomenon of „sorting-out“ (remember Wilsońs expt.). DAH postulates that - according to their expression and exposition of certain cell adhesion molecules (CAMs) on cell surfaces (e.g., cadherins or ICAMs) - similar cells would stick more or less strongly to each other. When agitated, more sticky cells would aggregate further inside an

aggregate, while those with weaker adhesion would be assembled around them. For instance, RPE cells, which are very sticky, after mixing with retinal cells would be found inside of the forming reaggregates. Since limb bud cells are even more stickier than RPE cells, they would arrange further inside than RPE (a.s.f.). Steinbergs work was directive in the search for CAMs. It should be noted that DAH can explain sorting-out of cells during the very beginning of a reaggregation process (time frame of minutes and hours), but what happens later within a reaggregate (after days and even weeks, e.g., in an organoid, see below) is much more complex than mere adhesion - it is *development in vitro*. This is what we have studied in much detail with retinal reaggregates over the coming decades.

6. Retinal spheroid technology – more than mere cell reaggregation

The works of Fujisawa, Moscona and Steinberg became the starting point of my own reaggregation experiments. The basic retinal reaggregation experiment is simple, starting by isolating the retina from 3 to 6 day old chick embryos. After dissociation of the tissue (applying trypsin) into dispersed cells, they are supplemented with appropriate medium and cultured in dishes (30 mm dishes, or 24 or 96 well plates). The cells are rotated on a shaker within a 37 °C incubator (note other suspension methods are available, e.g., rotation flasks, hanging drops, specified cell reactors). The speed of rotation, temperature and humidity, and - of course - substrate composition are all crucial (e.g., finding

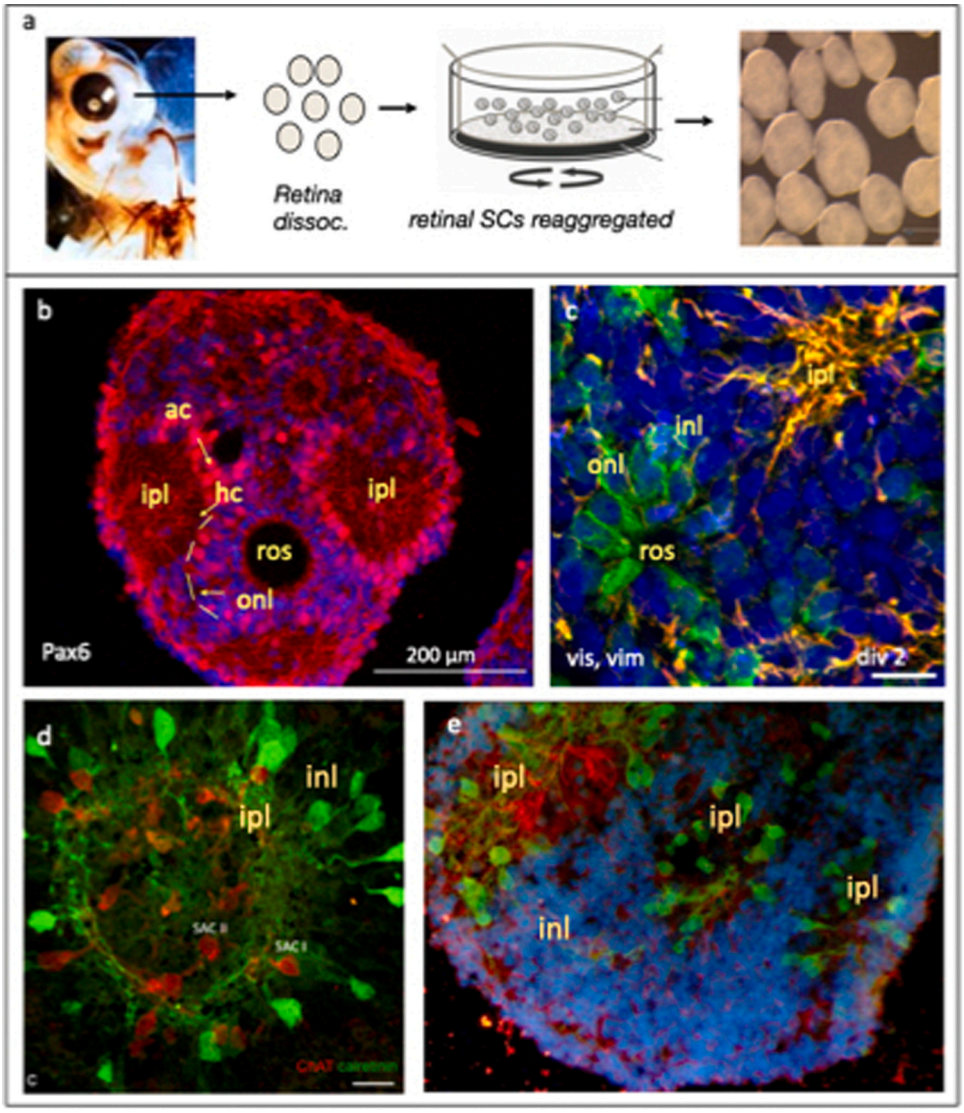

Fig. 2. Cells from the embryonic avian (a-d) and rodent (e) retinae reaggregated under suspension provided ideal 3D models to analyze processes of retinal tissue formation, eventually revealing the future potential of organoid technologies (see Fig. 3). (a) Scheme of method: embryonic chick retina is dissociated and cultured under suspension (cf. Fig. 4A, upper right) leading to irregular potato-shaped spheroids (right in a), called „rosetted retinopheroids". (b) Section of 8 day-old rosetted spheroid presents their general histology, including PR rosettes (ros) and large IPL-like areas (denoted as „ipl"); cells representing inner INL are Pax6^{+} (red); locations of an outer nuclear layer (onl) and horizontal cells (HCs) are indicated; DAPI is blue. (c) Higher magnification of a 2 day-old section presenting a photoreceptor (PR) rosette (ros) in the vicinity of an IPL-like area (ipl); PRs are visinin^{+} (green), precursors of Müller glial cells (MCPs) are vimentin^{+} (yellow); note MCP processes reaching through tissue. (d) Lamina formation in an IPL-like area (ipl): calretinin^{+} amacrine cells (ACs, green) send processes into ipl, there forming several parallel synaptic sublayers: these orient themselves along primary sublayers formed by pairs of ChAT^{+} ACs (red). (e) Rodent retinal reaggregates first organize their inner retina (in chick it is first the outer retina via PR rosettes). 10-day old section of rodent retinal spheroid (from Gerbil), which was triple-stained for calretinin (green), Ret-G7 (red) and DAPI (blue). PR rosettes are absent, while Müller cells (MCs, red) initiate IPL formation. Note several IPL-like areas are forming within one sphere; CR^{+} cells (green) are located on either side of ipl; their processes project into the ipl, turning at a sharp angle and arranging in parallel (from Bytyqi et al., 2007). Eventually, in these rodent spheres, highly laminar retinal organisation can be achieved (see Fig. 3e).

defined replacements for growth factors contained in sera represents a big challenge). Within minutes after seeding of cells in dishes, cells will reaggregate, form cell clumps and immediately begin to sort out. Within the first two days, potato-shaped solid reaggregates will form that will reach an optimal average size. Notably, these are not only products of reaggregation and cell sorting, but pronounced cell proliferation will occur, which is followed by cell differentiation; i.e., development and active tissue formation will ensue.

7. 3D-reconstruction of a retina – first retinal organoids in 1984

Besides the normally occurring irregular spheres (Fig. 2a, right), very rarely reaggregates could be detected that appeared as highly regular, translucent cell spheres (Fig. 3a-c; Vollmer et al., 1984; Rothermel et al., 1997). Their high degree of tissue organisation was evident even without a microscope. Since mutual interactions between developing retina and RPE had been reported (Moscona, 1960; Fujisawa, 1973), we included RPE cells in our retinal reaggregate cultures. As a result the percentage of highly organized spheres - which we called *stratified retinospheroids* - was dramatically increased. Indeed, cutting and staining of these complete spheres (Fig. 3b) revealed the anlage of all three cell layers corresponding in vivo to outer, inner and ganglion cell layer (abbrev. ONL, INL, GCL; labelled as onl, inl, gcl, Fig. 3b,c;). In these retinal spheroids, most retinal cell types begin to differentiate. For instance, outer segments of photoreceptors (PRs) are in the process of differentiation. Both synaptic layers (outer and inner plexiform layers; opl, ipl) are established. Ganglion cells are rare and soon will disappear, since they cannot establish connections with the brain. Note formation of a wide IPL, which in itself becomes highly stratified, composed of several parallel synaptic sublayers (see next). Further studies revealed that formation of such stratified retinal spheroids depends on presence of factors from the RPE, or alternatively, from radial glial cells. Led by our chicken work, we could achieve similar laminar spheroids with dispersed cells from the embryonic gerbil retina (rodent related to mouse and rat; Rieke et al., 2018). A Japanese group documented that the Wnt pathway is a lead driver to establish the laminar structure in chicken retinospheroids (Nakagawa, 2003). The chicken stratified retinospheroid was first published in 1984, and represents the first demonstration that a retina can be reconstituted *in vitro* from fully dispersed stem cells from the young embryonic chick retina (Vollmer et al., 1984), a structure which nowadays would be called a retinal *organoid*. The successful reconstruction of such highly laminar retinal tissues was indicative that *in vitro* reconstruction of a human retina might be feasible, as soon as appropriate numbers of human stem cells (SCs) would become available (Layer et al., 2001, 2002).

The potato-shaped spheres - called *rosetted retinospheroids* (Fig. 2a,

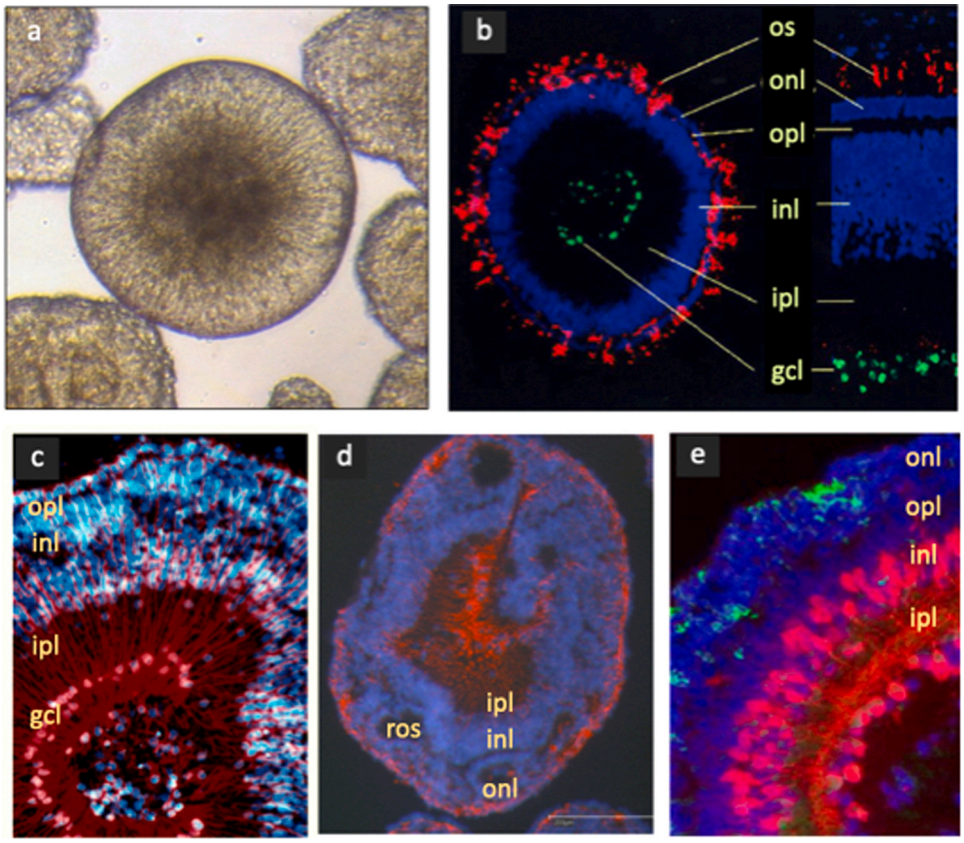

Fig. 3. Cells from the embryonic avian (a-d) and rodent retina (e) reaggregated under suspension provided ideal 3D models to achieve the first nearly complete laminar reconstruction of retinal tissue *in vitro* (Vollmer et al., 1984; for e, Rieke et al., 2018), foreshadowing the future potential of organoid technologies. (a) Translucent, highly regular spheroids from avian retinae - called *stratified retinospheroids* - are produced under the influence of RPE or Müller glial cells (see text). (b) All layers are reconstructed, as shown on a section of a 10 day-old stratified spheroid (left), as compared with a normal retina (right). PRs are stained red, DAPI is blue, few ganglion cells (gcl) plus displaced ACs are color-coded in green. (c) Radial processes of MC precursors (F11 antibody, red) reach through entire width of stratified spheroid, separating individual cell columns from each other (see Willbold et al., 1996). (d) A rosetted spheroid during the transition into a stratified spheroid. Note many PR rosettes under surface of spheroid, which will soon fuse to form a coherent PR layer. The opl fuses at several points; similarly, the inl is almost complete; in center forms a wide ipl. Note strongly stained inner and outer endings of MCs (red). (e) Nearly complete stratified retinospheroid from embryonic rodent retina, resulting from initial exposure to Wnt3a, followed by RPE supplementation. Numerous CR^{+} ACs (red) and their counterparts near the inner core send processes into the ipl, where they navigate laterally. For details see Rieke et al., 2018. RPE, retinal pigmented epithelium; picture in (d) is from G. Bachmann, unpublished.

right) - are predominant in chicken retinal 3D cultures. They will form in absence of any specified factors and can be produced easily at high numbers. They are irregular in shape, appear less complete than stratified spheroids, but - which is often neglected - are not less relevant to answer basic science questions. Since processes happening in spheroids are more than just sorting-out, cryptic features of tissue development can be disentangled by analysing these irregular reaggregates. In short, rosetted spheroids are comprised of retinal tissue modules representing the outer (rosettes of PRs) and inner retina (INL, IPL; in Figs. 2, 3 labelled as „inl" and „ipl"). While not being as complete as fully stratified spheroids, in this simple and highly artificial cell sphere the cells have a drive to differentiate and organize themselves into some kind of retinal tissue. By manipulating these spheroid cultures, many basic questions can be tangled. For instance, will all the different retinal cell types differentiate, which ones are missing under which culture conditions? Will they arrange correctly within the 3D space, what conditions are required to do so? Will synaptic layers form and become organized to which degree, can they form synapses with each other? Indeed, a first glimpse on stained sections of a rosetted spheroid looks like chaotic (Fig. 2b). But evidently, these spheres present some structure, characterized by cellular rosettes (see below, Figs. 2b, 3d), but also by wide cell-free spaces (IPL-like spaces, Fig. 2b-d). In between these two outstanding areas, more or less organized cellular spaces present INL-like areas (Fig. 2b,c; see below); hence all major cell types are found in rosetted spheroids. For achieving retinal tissue structure in the first place, radial Müller glial cells (MCs) play leading roles, which can be analyzed in both types of retinospheroids (rosetted vs. stratified). Expressing early MC markers but long before glutamine synthetase (i.e., their typical cell marker) occurs, long processes of immature MCs reach through the width of aggregates (Fig. 2c). Better visible in stratified than in rosetted spheroids, they separate individual cell columns from each other (Fig. 3c), and hence structurize the developing 3D tissue space (Willbold et al., 1996). Occasionally, a transition of the rosetted state into a laminar spheroid state could be detected (Fig. 3d). Rosettes near the surface would open up and fuse, as would sections of IPL and INL. Notably, strongly stained endfeet of MCs stood out, indicative of their active role in this revolving histogenetic process. Revealing their immature state (a state between neuroepithelial and undifferentiated glial cell), MCs (and/or their precursors, MCPs) can - under certain conditions - not only proliferate and induce formation of stratified spheroids, but remarkably, one such immature glial cell can divide into one Müller glial cell and one early amacrine cell (Bachmann et al., 2019). Hence in these model systems MCPs exert functions of pluripotent stem cells. This pivotal capacity of MCs as observed in avian spheroids was again documented during formation of human iPSCs into 3D retinal organoids (cf. Finkbeiner et al., 2022). As mentioned above, cell rosettes are conspicuous hallmarks of rosetted spheroids, within which - using immunohistochemistry - all types of avian photoreceptors could be detected (note: PRs began to form outer segments, but never reached full maturity). Interestingly, their relative ratios were varying, depending on applied culture conditions (e.g., comparing rosetted vs. stratified spheroids). Thus, the composition of different classes of PRs is not genetically fully determined, but depends on their environments (Layer et al., 1997; Rothermel and Layer, 2001; Jacob et al., 2005; Volpert et al., 2009). Notably, along with PR differentiation, rosetted spheroids secreted melatonin in an oscillating manner in response to an applied light-dark light cycle (12 h/12 h), achieving maximal melatonin levels in the dark. As a corollary, two key enzymes of its synthesis were regulated accordingly (Willbold et al., 2002). Thus, spheroids can establish meaningful physiologic responses, which warrants their usefulness as analytical *in vitro* assay systems (see below). Similar to the outer retinal components, differentiation of the inner retinal half in rosetted spheroids is as remarkable. It is represented by cells of the INL together with cell-free spaces representing the IPL (Fig. 2b,c). Cell processes originating from INL-like spaces begin to organize a somehow stratified IPL. Thereby, processes from amacrine and horizontal cells project into quite large areas. Corresponding with the inner synaptic layer IPL, these become filled with neuritic and glial materials. To give an example of complex processes of synaptogenetic differentiation occurring within rosetted retinal spheroids, an advanced network formation within its IPL area was revealed (Bachmann et al., 2019). In vivo, a particular type of cholinergic amacrine cells (so-called *starburst amacrine cells, SACs*) are placed on both sides of the IPL; their neuritic projections form two parallel synaptic layers within the IPL. These represent the first synaptic sublaminae forming during IPL maturation, along which many more synaptic sublayers will orient themselves, and thus will become arranged in space. Similarly in rosetted spheroids, several parallel synaptic sublayers are established within an IPL space (Fig. 2d). Amazingly, the very first synaptic sublayers of the IPL are well reconstructed *in vitro*. Thus these studies revealed how the complex process of IPL sublamination is initiated and directed by cholinergic SACs in simple and artificial *in vitro* cell culture models.

As mentioned above, reconstruction of retinal tissue from dispersed cells was not only possible from chicken avian embryonic retinae, but also from mammalians, however significant developmental differences were noted. While with avians, precursors of photoreceptors, i.e., cells belonging to the outer retina were the first to organize within reaggregates, while in gerbil retina the leading role was taken over by cells from the inner retina (Fig. 2e). The relatively late differentiation of PRs in rodents (as compared with avians) may explain this different sequence of tissue differentiation. Thus, in absence of a leading cell type (PRs in early rodent retina) other cells (here from inner retina) are able to initiate and direct formation of a 3-fold laminar retinal tissue (Bytyqi et al., 2007; Rieke et al., 2018). A general insight provided by reaggregates was, that it is not the genome (the genes) only that directs formation of a tissue, but as much the surrounds, or, in other words: formation of a tissue is due to the action of genes plus time-space-dependent selforganisation, together leading to emergent effects, which drive and direct different cells in combination to reach a certain goal (in this case a „retina-like tissue spheroid"). Their diverging parameter settings are decisive for a successful tissue reconstruction, yet - unfortunately - cannot be fully predicted, but need to be established for every new tissue situation (note: in the future KI technologies may help with this complication). Such multi-faceted properties of *in vitro* tissue formation could be conceived as „many roads leading to Rome" (Layer, 2019). As a side note, such target-directional developmental effects foster the recent quest for an extension of the standard evolutionary theory (*Evo-Devo, teleology;* Layer, 2022).

8. Organoids based on two technologies: 3D-reaggregates & human stem cells

Up to the late nineties this work on retinal tissue reconstruction *in vitro* remained an entirely academic endeavor. Only few research teams were working with reaggregate models, i.e., not more than five on retinal spheroids, a few others on kidney, liver and brain. While interest for this work remained negligible, cooperations with engineers already heralded their relevance for application in health and disease. For instance, retinal spheroids from gerbil retina raised in a multi-container bioreactor system provided spheroids of similar sizes, whereby in each microcontainer (only) one spheroid was forming (Fig. 4A). Notably, agitation of the container system was not necessary, rendering it suitable as a high through-put assay system for pharmacology and toxicology research (Rieke et al., 2008). An even more uniform shape of spheroids can be generated using arrays of canonical agarose microwells (Thomsen et al., 2018). In these micromolded hydrogel-embedded microwells culturing of different cell types becomes feasible; e.g., tumor cells when co-cultured with bone marrow stromal cells formed larger spheroids. Another approach was designed to analyse non-invasively growth and morphologic changes in non-adherent tumor spheroids by impedance spectroscopy, allowing high throughput development of anti-tumor drugs (Kloss et al., 2008). Furthermore, in probing for possible

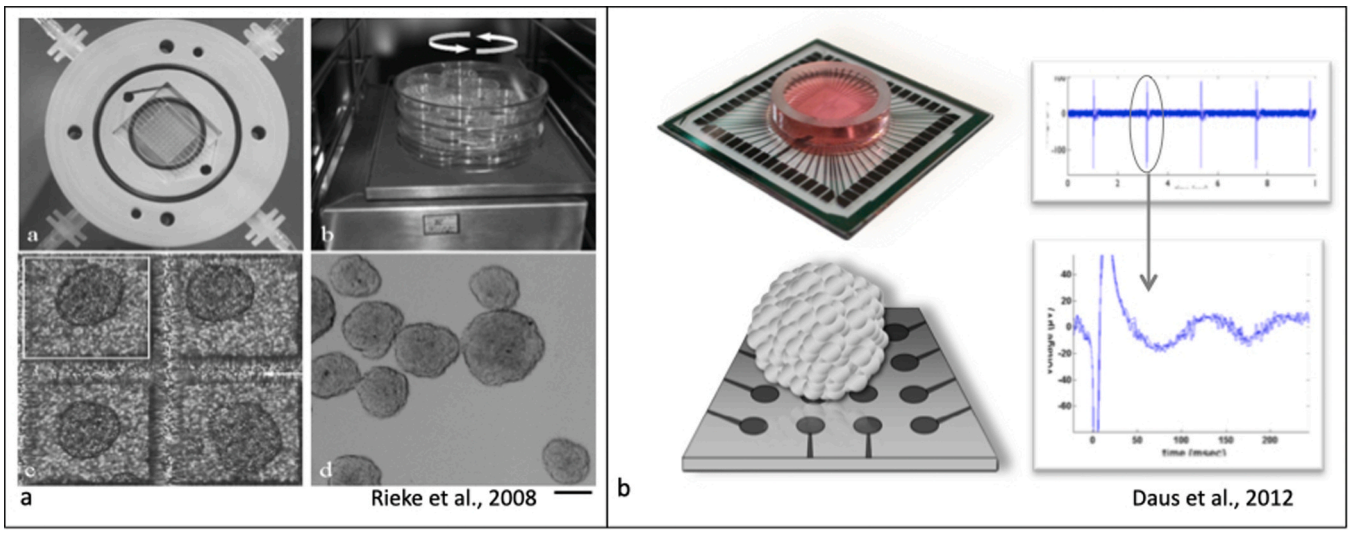

Fig. 4. Developing organoid assay platforms - from cells cultured in simple rotating plastic dishes to flow-through culture systems with high throughput. A, left) individual spheroids from gerbil (rodent) retinas (lower) are cultured in a flow-through microcavity system (upper). A, right) large numbers of spheroids from gerbil embryonic retinas (lower) are cultured on a simple gyratory shaker in 35 mm dishes (upper). B, upper left) cell reaggregation culture system containing spheroids from embryonic avian cardiac myocytes placed on a MEA chip. Spheroids can be treated pharmacologically, or, exposed to a constant EMF (details, e.g., for exposure system, data management, variance of cell-electrode coupling, see Daus et al., 2011). B, lower left) Single spheroid harvested from shaker (upper) is placed on single electrodes for analytical measurements; B, right) action potentials from one spheroid are monitored, corresponding with optically measured contraction rate (not shown). Pictures from Rieke et al., (2008) (A) and from Daus et al., (2011), (2012) (B).

deleterious effects of mobile phone radiation, exposure to defined EMFs was tested on spheroids, as produced from reaggregated avian cardiomyocytes. The rate of their visually detectable contractions („heart beats“) corresponded with the rate of measured action potentials (Fig. 4B). Again, pharmacological interferences with cardiac physiology could be reliably analysed in this system (Daus et al., 2011, 2012). Thus at the turn of the century, revival of reaggregation methods, in particular their applicability in medical research and therapy was on its brink (Layer et al., 2001, 2002). Indeed, the intention of stem cell biology was to get means to manipulate pluripotent cells from human blastocysts (i.e., stem cells having the potential to differentiate into all somatic cells) such that differentiated cells from all three germ layers could be produced. This aim was eventually achieved by intense research with embryonic stem cells (ESCs) from mouse and then from human blastocysts by learning how to molecularly direct such cells towards differentiation into many types of cells of the mammalian body. Improved procedures of handling cells were introduced, whereby typically some steps of suspension cultures were decisive (sometimes a combination of 2D standing and 3D suspension cultures was applied). Thereby, 3D-cultures came strongly back on the stem cell agenda. The issue was not only limited to produce a certain cell type from ESCs, but to aim at developing entire tissues, or even organs from them. The rapidly growing field of *Tissue Engineering* (TE) was standing on two legs - i) manipulation of ESCs (preferentially from human) by use of ii) 3D-reaggregate cultures. Indeed, recent progress of organoids as physiologic, anatomical or pathologic assay systems, or, as TE platforms is widespread. For instance, stem cell-derived cardiac organoids (also in combination with other cells; e.g., endothelial) can be applied under various settings (e.g., encapsulated in a synthetic or natural extracellular matrix, as microfluidic bioreactors, etc.), and are expected to promote cardiovascular basic research and drug discovery (Joddar et al., 2022). Referring to brain research and diseases, so-called assembloids represent reaggregated organoids from pluripotent stem cells, which - when kept alive over months - allow investigating long-range 3D neural network establishment, including their electrophysiologic analysis (Miura et al., 2022). And this list needed many more examples.

9. Invention of iPSCs — a breakthrough in stem cell biology

Albeit remarkable progress was made with ESCs in the laboratory, there were deterrent problems with their envisaged medical applicability. The number of available cells from blastocysts was low, and more importantly, there were unsurmountable ethical problems: from where could one get large numbers of human ESCs to be used for large scale research, and even more so, for tissue/organ regeneration and transplantation? It was clear to everybody, that large-scale use of ESCs from aborted fetuses were not a choice. No one really would invest into such a doubtful endeavor. The solution was brought about in 2006 by Shinya Yamanaka with his invention of the so-called *reprogramming* of adult fibroblasts to produce so-called *induced pluripotent stem cells* (iPSCs; Takahashi and Yamanaka, 2006). To bring their differentiated state back to an embryonic state comparable to ESCs, i.e., a combinatorial expression of a few genes was required (e.g., KLF4, SOX2, c-Myc, Nanog, Oct3/4, Lin-28). The advent of this ingenious technology changed everything overnight, it was a major move forward in stem cell biology, and importantly: the ethical problem of producing pluripotent stem cells at large scale was solved. Now the way for Tissue Engineering became feasible, the door for *Regenerative Medicine* in general was pulled wide open.

10. From cellular spheroids to human retinal organoids: “optic vesicles” from iPSCs

Again pioneering that route, the vertebrate retina with its small number of different cell types and its distinct threefold lamination was an obvious target for stem cell biologists and tissue engineers. There are a number of groups which have made remarkable progress into production of human retinal spheroids from pluripotent stem cells (e.g., Eiraku et al., 2011; Meyer et al., 2009; Cowan et al., 2020; Finkbeiner et al., 2022), although a complete reconstruction of human retinal tissue has not yet been achieved. Based on the significance of the Wnt pathway to establish a full lamination in retinal spheroids (Nakagawa, 2003), Sasaiś group succeeded in producing the first eyecup-like organoids from mice iPSCs (Eiraku et al., 2011). David Gammś group (Madison WI/USA) produced similar „optic vesicles“ from human PSCs (incl.

iPSCs), spherically shaped organoids presenting a remarkable degree of differentiation (Meyer et al., 2009). Their outer retinal part appeared highly differentiated, including an advanced formation of photoreceptor (PR) outer segments, while the inner retina remained incomplete. In particular, a sublamination of the IPL - as has been detected for avain spheroids - was absent. Their detailed analysis revealed which genes at which temporal stage are required during formation of optic vesicles in order that iPSCs really find their way towards forming retinal cells and in the end an organized tissue. During a long culture period of several weeks, the expression of particular genes mark decisive decision points, e.g., developmental stages defining the eye field, the optic vesicle stage, and the division between retina and RPE formation. Notably, the detected genetic cascades and the time-course *in vitro* are comparable to those of the *in vivo* development of a human retina. Notably, development of human optic vesicles leads to similar three-dimensional structures as produced from avian and rodent retinal precursor cells (Fig. 3). This denotes that research on human retinal tissue reconstruction could take advantage from discoveries of avian and rodent reaggregate studies.

11. Brain organoids presenting specific brain regions

After the invention of iPSCs by Yamanaka, the research field from stem cells towards TE sky-rocketed. As a corollary, human organoids from iPSCs became a hot topic in stem cell biology and TE fields. For instance, iPSC-derived organoids were successfully raised from lung, intestine, prostate, kidney, eye and brain tissues (Huch et al., 2017). One of the early projects that made it into the limelights came from Jürgen Knoblichś group in Vienna, where Madeline Lancaster produced highly structured neural organoids from iPSCs, in which individual brain regions were reconstructed. After appropriate genetic manipulation, such human CNS organoids promised to become models for analysing - *in vitro* - certain brain diseases (e.g., dementias) and to develop medications for them. As an important further advantage, animal experimentation could be replaced by organoid technologies to a large extent.

12. *In vitro*-production of man? “Sheefs” — synthetic human embryo models

In the meantime, not only organoids modeling brains or liver, but reproduction of whole embryos were inspiring the actions of stem cell researchers. Following reaggregation of naive mouse pluripotent ESCs on a gyratory shaker and their further electronic control in an *ex utero* device allowed formation of mouse organoids, which reached post-gastrulation stages up to E8.5, including advanced embryonic and extraembryonic structures (Tarazi et al., 2022). Are we going to see humans that are derived not from a father and a mother, but from one somatic cell of one particular person? Hopefully never, we all would say. But what sounds like Golem (an old Jewish legend of artificially making man), such things indeed now seem to emerge at the horizon. Most recent headliners come from Magdalena Zernicka-Goetz in London about production of "synthetic human embryos" (*sheefs*; Apostolou et al., 2023). Production of so-called *Gastruloids* or *Sheefs* from hiPSCs have been reported. These are organoids resembling human gastrulae (up to the stage of nidation, 14 days p.f.), which are characterized by formation of a primitive streak. As a notable advantage, human sheefs open a way to analyze the very first steps of human development, which cannot be approached by other means (note: this applies as much to animal research; Kar et al., 2021; see below). However from an ethical point of view this technique remains rather anbiguous, since sheefs are human embryos not derived from a zygote (e.g., the result of fertilisation of an egg by a sperm), but are derived from an iPSC; i.e., not from the genes of two persons, but only from one. At the moment nobody can foresee whether these synthetic human embryos could survive in an uterus, and eventually be born as a human being. Clearly this investigative route requires strict legal regulations.

13. Organoids — hopes, hypes and fears

Our walk through more than a century of stem cell biology ends with the triumph of regenerative medicine. Huge medical progress can be foresighted. Based on production of organoids from iPSCs (both human or animals), four major fields of applications are in sight: 1. Providing *in vitro* models to analyze basics of human diseases, useful as human assay systems for drug development, even development of patient-specific assays seems feasible. Notably, organoids can be applied on electronic chips, called *organ-on-a-chip* (or, *lab-on-chip*), providing high throughput platforms with wide applications and massively reducing animal experimentation (Ma et al., 2021 for review; Rieke et al., 2008, Daus et al., 2012; Fig. 4). 2. Novel analytical and production technologies for pharmacology, toxicology, agriculture, nutrition and environmental protection become amenable, i.e., basic and translational fields that are already fully flourishing. The great values that organoid technologies offer for research and innovative productions in agriculture and food technologies appear almost endless (for review, Kar et al., 2021). For instance, organoids representing nearly all organs of major livestock and pet animals will provide analytical test platforms, e.g., to investigate interactions of natural microbiomes, or, of potential pathogens with gut epithelia, evaluate supplemental or toxic feed/food additives, provide new concepts of phenotypic breeding (i.e., traits could be tested and selected in organoids derived from animals with particular disease resistances, feed efficiencies, asf.). 3. Production of tissues or whole organs for transplantation/implantation. This field is more challenging, since many questions remain open; e.g., tissue shape management (i.e., how to restructure a spherical organoid into the desired natural form, incl. bioprinting methods (Dey and Ozbolat, 2020; e.g., a retinal spheroid into a flattened retina); interactions with/integration into other tissues/organs, blood supply, immune reactions, methods of implantation, etc.; and finally, 4. massive reductions and replacements of animal experimentation in basic and translational sciences will represent not the least relevant progress of organoid technologies. Taken together, no technological innovation can bring forward the holy grail, and unforeseeable misuses (medical, ecological, or military) can never be completely avoided. Thus, ethical fears must be taken seriously, some present hypes will lead into dead ends, yet some major hopes of organoid technologies appear not only valid, but are already materializing.

A full special issue has been devoted to the topic of organoids in the leading journal *Development*. This medical progress has its roots - like often - in basic biological sciences. It may appear that the progress appeared upruptly like phoenix from the ashes, but - as was shown here - this revolutionary technique was made possible through more than a century of research based upon 3D suspension cell cultures. People working on simple animal model systems, using simple methodologies - sponges, polyps and worms, followed by avian and rodent embryos - have raised very basic questions of cell biology. First analyzed in reaggregates of simple animals and then from several tissues of vertebrates (with the retina as an outstanding model), it was discovered that 1) cells communicate and segregate self from nonself (sort out); 2) following reaggregation there occurs development *in vitro*; 3) thereby, stem cells divide and differentiate, to reach 4) a far-advanced tissue formation. Furthermore, neural cells can 5) form integrated networks (incl. synaptogenesis, lamina formation). Strikingly (6), tissue reconstruction can be reached along different histogenetic pathways (e.g., retina from avian vs. rodent retina). All this is made possible (7), since genetic cascades are regulated - both *in vivo* and *in vitro* - by temporal and spatial limitations, i.e., environmental conditions, leading to emergent self-organisational effects (Layer, 2019, 2022). Thus, elaborate research analysing stem cell behaviour in 3D suspension cultures paved a long way to eventually lead to a tremendous progress in medical research. Hence it should never be neglected that medical progress depends to a large degree on biological and - similarly important (not dealt with here) - technological progresses.

CRediT authorship contribution statement

Paul Gottlob Layer: Writing – review & editing, Writing – original draft, Supervision, Project administration, Formal analysis, Conceptualization.

Declaration of Competing Interest

I have no interests to declare.

Data availability

Data will be made available on request.

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