Biophysical regulation of cell reprogramming

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Abstract

Induced pluripotent stem (iPS) cell reprogramming and direct reprogramming are promising approaches for disease modeling and personalized medicine. However, these processes are yet to be optimized. Biomaterials are increasingly integrated into cell reprogramming strategies in order to engineer the microenvironment, improve reprogramming efficiency and achieve effective in situ cell reprogramming. Although there are some studies on the role of biomaterials in iPS cell reprogramming, their effect on direct cell conversion has not been fully explored. Here we review the recent advances in the use of biomaterials for iPS cell reprogramming and direct reprogramming, with a focus on the biophysical aspect. We further highlight the future challenges and directions of the field.

Differentiation, reprogramming and direct reprogramming

Stem cells have the ability to differentiate and self-renew. Cell differentiation is the commitment of a relatively more potent cell towards a more defined lineage or cell type. Up till the first half of the 20th century, the prevalent view was that mature cells were permanently locked into a differentiated stage and could no longer be reverted into an immature, more pluripotent state. It wasn't until 1962 that John Gurdon first reported that the reverse process is achievable by demonstrating that nuclei from terminally differentiated *Xenopus* intestinal epithelial cells were capable of generating full functional tadpoles upon transplantation into enucleated oocytes [1]. This reverse process is now known as reprogramming.

Apart from somatic cell nuclear transfer, reprogramming was previously also achieved by cell fusion and the use of transcription factors. In 2006, Takahashi and Yamanaka distilled the four core transcription factors *Oct4*, *Sox2*, *Klf-4* and *c-Myc*, capable of reprogramming any cell back to the pluripotent state and termed these cells as induced pluripotent stem cells (iPSC) [2]. This discovery opened up the possibility of generating any cell type from a somatic cell by first reprogramming them back to an induced pluripotent stage and then differentiating them towards the target cell type, a process known as directed differentiation. Since then, direct reprogramming has emerged as a new method to manipulate cell fate. Direct reprogramming, as the name suggests, is the direct conversion of a mature cell into a completely different cell type without passing through the intermediate induced pluripotent stage (Figure 1). Multiple studies have shown that this method can be used to achieve a wide range of target cell types [3–8]. In addition, it is usually a shorter and more straightforward process compared to iPSC

reprogramming followed by differentiation. More importantly, it circumvents the teratoma-forming potential typically associated with the intermediate pluripotent stage for iPSC. It is currently one of the most promising approaches for personalized and regenerative medicine and for the generation of patient specific disease models for drug screening. In addition, it allows for monitoring of human disease progression such as Alzheimer's which traditionally could only be accessed at the end-point.

While early studies commonly used lentiviral and retroviral vectors for the delivery of transcription factors, other non-integrating delivery methods of achieving iPSC and direct reprogramming such as episomal plasmids [9], mRNA [10], micro-RNA [11,12], proteins [13] and small molecules [14] are increasingly being explored and adopted. More recently, biomaterials have also been found to play an important role in regulating the reprogramming process (Figure 1).

Influence of the microenvironment and biomaterials on cell fate

The microenvironment is known to influence cell fate and behavior. In turn, cells interact with and remodel their niches. A comprehensive understanding of this bidirectional interaction between cells and niches is crucial to guide our design of next-generation biomaterials and to accurately control cell fate in a precise and predictable manner. The properties of biomaterials can be largely separated into two categories – biophysical and biochemical properties. The former mainly consists of biomaterial stiffness, topography, ligand density, ligand patterning, three-dimensional (3D) environment, thermosensitive properties, shear stress, mechanical stretch and the size and mechanical property of micro/nanoparticles, while the latter consists of the composition of extracellular matrix (ECM) or immobilized ligands, soluble chemical factors released from drug carrier or the substrate, and the types of drug delivery vehicles (illustrated in Figure 2). Much work in the last two decades have established how biophysical factors can influence cellular processes, such as morphology [15,16], proliferation [17], migration [15,18] and differentiation potentials [19–21] of various adult stem cells, by providing instructional cues that modulate gene and protein expression to regulate cell state. However, how biomaterials regulate cell reprogramming is much less understood. In this review, we focus on biomaterials and their effects on cell reprogramming into iPSC and direct reprogramming into specific cell types.

Biomaterials in iPSC reprogramming

Traditional lenti- and retroviral-based vectors used in the delivery of reprogramming genes can result in random integration of the viral DNA into the host genome potentially leading to oncogene activation. This eliminates the use of viral vectors in any *in vivo* applications. Using biomaterials in the form of biodegradable cationic polymer PEI-coated super paramagnetic nanoparticles for transient iPS gene expression, Lee *et al.* successfully generated mouse iPSCs at high efficiencies compared to other non-viral systems [22]. Moreover, over 60% of the resultant iPSCs produced from this nanofection technique were free from integrated exogenous DNA.

The biochemical components of biomaterials, such as ECM or other ligands, can undoubtedly influence iPSC reprogramming. For example, chemically-defined self-assembled monolayers

displaying heparin-binding peptides derived from vitronectin promote cell adhesion through interaction with cell surface glycosaminoglycans and were found to be most effective in long term propagation and maintenance of pluripotency in human iPSCs [23]. Furthermore, collagen has been found to be a barrier to reprogramming and decreasing collagen through suppressing gene expression or collagenase treatment in mouse fibroblasts improves reprogramming efficiency [24].

The biophysical aspects of substrates on which adherent cells are reprogrammed have also been reported to play a critical role in directing the process. As an example, Downing *et al*. elucidated that biomaterial topography in the form of parallel microgrooves and nanofibers directs cell morphology to enhance reprogramming efficiency, i.e., the number of $Nanog^{+}$ colonies, through mechanomodulation of the epigenetic state of mouse fibroblasts [25]. Suppression of HDAC2 activity coupled to the upregulation of WDR5 expression respectively increased histone acetylation and methylation. The topographic cues also enhanced mesenchymal-to-epithelial transition (MET) and thus iPSC reprogramming. Cordie *et al*. further highlighted the differential effects of nanofiber polymer identity to enhance MET of human fibroblasts during reprogramming [26].

Besides biomaterial topography, recent works have unraveled the contribution of stiffness in fibroblast to iPS reprogramming. 0.1 kPa polyacrylamide gels induced MET and increased expression of Oct4 and Sox2 on adhered cells during reprogramming relative to the 20 kPa gels [27]. Similarly in an earlier study, softening of substrate stiffness has been shown to achieve reprogramming in HEK-293 cells simply through changes in actin force without any transcription factors [28]. More recently, Caiazzo *et al*. explored the possibility of a synthetic "reprogramming niche" intended to more accurately tune and mimic the stiffness, degradability and the biochemical composition of the *in vivo* microenvironment [29]. Upon optimizing PEG-based hydrogels to generate a 3D culture system for iPSC reprogramming, the authors observed improved reprogramming efficiency on gels with stiffness ranging from 300-600 Pa and enriched with laminin or Epcam, suggesting the contributing effects of substrate stiffness and cell-hydrogel ECM interaction on the reprogramming process. Most importantly, the authors found that the physical confinement exerted by the 3D system on cell morphology facilitated a greater than 2-fold increase in mouse and human iPS reprogramming efficiency. This occurred through accelerated MET and epigenetic remodeling with increased Histone 3 acetylation and methylation, in agreement with previous findings [25]. This system also selects for colony-forming iPSCs and limits the proliferation of non-colony forming cells. Apart from enhancing reprogramming efficiency, this proof-of-concept study also provided the model system for future studies to modularly tease apart the interaction between biophysical factors and genetic manipulation from transcription factors.

Interestingly, Luni *et al*. highlighted a similar concept of confinement when they discovered that a microfluidic culture system can dramatically increase the efficiency in synthetic mRNA-driven human iPSC reprogramming by up to 50-fold under xeno- and feeder-free conditions [30]. This remarkable effect is reportedly in part due to the decrease in the concentration of soluble signaling molecules in the culture media caused by the downscaling process. The effect of physical confinement from the microfluidic environment was also suggested to be another main contributor to the enhanced efficiency through extracellular accumulation of secreted factors

produced by the cells already undergoing reprogramming which likely provided cues for other surrounding cells to do the same.

The confinement effect discussed above is not expected to be found in normal 2D culture. In fact, Sia *et al*. conversely reported enhancement in the reprogramming efficiency of mouse iPSC with dynamic culture applied at mid to late phase of reprogramming [31]. The authors attribute this to convective mixing that prevents cell cycle arrest as cells reached confluency. Interestingly, suspension culture with stirring has been shown to be a scalable method to reprogram fibroblasts into iPSCs with improved efficiency [32,33]. In this case, stirring is important for maintaining aggregate size and may suppress differentiation through fluid shear stress.

More recently, electromagnetic fields have emerged as another biophysical factor that can influence cellular behavior and iPS reprogramming. Baek *et al*. demonstrated that exposure to extremely low-frequency electromagnetic fields (EL-EMF) could not only significantly enhance the reprogramming efficiency of converting mouse fibroblasts into iPSCs but more importantly, it could replace transcription factors from the reprogramming cocktail as functional iPSCs were derived from the forced expression of only Oct4 with EL-EMF exposure [34]. Remarkably, EMF could dynamically regulate epigenetic changes (i.e. accumulation of H3k4me3) through the activation of the histone lysine methyltransferase MII2.

Apart from influencing the initiation of reprogramming, biomaterials have also been studied for their role in iPSC maintenance and differentiation. For example, graphene preferentially maintained mouse iPSC in the pluripotent state and furthermore seemed to suppress endodermal differentiation whereas graphene oxide expedited iPSC proliferation and enhanced endodermal differentiation compared to ectodermal or mesodermal lineages [35]. Moreover, a thermoresponsive 3D PNIPAAm-PEG hydrogel scaffold has also been developed for long-term efficient and scalable human iPSC expansion [36]. The synthetic, chemically well-defined hydrogels reduce cell aggregation and insulates iPSCs from shear forces that are otherwise associated with cytotoxicity in suspension culture [37].

Biomaterials in direct reprogramming

Within the realm of direct reprogramming, an interesting observation is that direct reprogramming of fibroblasts into cardiomyocytes *in vivo* using cardiogenic transcription factors *Gata4*, *Mef2c*, and *Tbx5* is more efficient than that *in vitro* [38], clearly suggesting that elements exist within the microenvironment that can impact this process. Biomaterials offer an advantage as they can be engineered to control ECM ligand type and density and thus influence the reprogramming process. Using a different protocol whereby cells are partially reprogrammed through the inducible control of OSKM and, without reaching pluripotency, directed to differentiate into cardiomyocytes, Smith *et al*. demonstrated that engineered poly(ethylene glycol) (PEG) hydrogels presenting high concentrations of laminin and RGD peptide were able to yield twice as many cardiomyocyte-like cells from mouse fibroblasts compared to the Matrigel-coated culture dish [39]. Moreover, it was elucidated that the inclusion of RGD peptides provided better long-term cell adhesion, enhanced proliferation and did not interfere with the reprogramming process. Similarly, Kong *et al*. reported that cardiac reprogramming was more efficient in gels composed of both fibrin and collagen. Defining the efficiency as the percentage

of contractile colonies out of the total number of cell colonies, they showed that the efficiency was correlated to an optimal concentration of collagen [40].

Microgrooves can induce cell alignment to mimic cardiomyocyte organization *in vivo*, and enhance the directed differentiation of cardiac progenitors into cardiomyocyte-like cells [41]. Sia *et al*. investigated how different biophysical factors could affect the efficiency of directly reprogramming mouse fibroblasts into cardiomyocytes using the specific cardiogenic transcription factors *Gata4*, *Mef2c*, and *Tbx5*. It appeared that microgrooves, but not cyclic uniaxial stretch or substrate stiffness, improved the yield of beating cardiomyocytes [42]. In addition, transcriptional factor Mkl1 was identified as a mechanotransducer that was activated on microgrooves, and together with AcH3, played a role in the topography-enhanced reprogramming.

Kulangara *et al*. examined the effects of directly reprogramming mouse embryonic fibroblasts into neurons on various topographical cues in conjunction with lentiviral delivery of *Ascl1*, *Brn2*, and *Myt1l.* Neuronal beta-III tubulin (Tuj1) positive induced neurons (iNs) were generated on polystyrene substrates consisting of smooth controls, microgratings and microposts [43]. Interestingly, the micrograted substrates were found to enhance the conversion efficiency (percentage of iNs out of total fibroblasts) and iN purity by approximately 1.5-fold. Topography was shown to influence neuronal properties, such as neurite branching, and could yield functional iNs that showed no apparent differences in electrophysiological characteristics when compared to iNs generated on smooth surfaces. Additionally, it has also been demonstrated that nanogrooved substrates can promote the direct reprogramming of mouse embryonic fibroblasts into induced dopaminergic (iDA) neurons, whereby the derived neurons expressed dopaminergic markers and acquired a mature neuronal phenotype. The increase in the conversion efficiency with the nanogrooved substrates was attributed to specific histone modifications (i.e. H3k4me3) and the upregulation of mesenchymal-to-epithelial gene expression [44].

As focus shifts to find alternatives to viral-mediated gene deliveries or cumbersome protein transduction methods, radio electric conveyed fields are proving to be a new viable option for direct reprogramming strategies. For instance, Maioli *et al*. demonstrated that it was possible to use a Radio Electric Asymmetric Conveyer (REAC) to deliver radio electric conveyed fields and promote the conversion of human skin fibroblasts into cardiac, neuronal and skeletal muscle lineages without the use of exogenous transcription factors [46]. The authors showed that REAC treatment was able to induce the expression of genes specific to multiple lineages within a few days of stimulation and this was found to be regulated by the transcriptional induction of the NADPH oxidase subunit Nox4. Similarly, it was shown that REAC could regulate stemness-related genes (e.g. Oct4, Sox2, cMyc, Nanog and Klf4) in a biphasic manner that prevented persistent reprogramming into a pluripotent state. The biophysical factors and the direct reprogramming systems discussed in this section are summarized in Table 1.

Limitations and future of the field

While ESC and iPSC derived cells are expandable and iPSC derived cells offer the advantage of being autologously sourced, careful consideration and purification steps must be taken into account to avoid risks of teratoma formation and tumorigenicity that may arise from the long

term culture conditions of such cells. The process of iPSC derivation, expansion and directed differentiation is lengthy and costly. Alternatively, direct reprogramming strategies have been shown to bypass the pluripotent state and provide a faster method of obtaining somatic cells than iPSC reprogramming. However, challenges still exist. The conversion efficiency is still relatively low, and the use of viral-based gene delivery limits their use for therapeutic purposes. Additionally, these reprogramming strategies could lead to partially reprogrammed cells that could potentially pose risks for cell transplantation applications. Recent studies have shown it is possible to use chemical cocktails to directly convert fibroblasts into cardiomyocytes and neurons [46–48]. To overcome the low efficiency, biomaterials can be used to engineer the *in vitro* niche and to realize the controlled release of the chemical cocktails *in vivo* for in situ reprogramming. Moving forward, one may design smart materials with tunable physical properties, such as matrix stiffness, degradation/remodeling and hydrogel chemistry.

To date, most direct reprogramming strategies involving biomaterials are performed *in vitro* using mouse models. While biomaterials and biophysical cues may enhance the reprogramming process, they are not absolutely required in the *in vitro* studies, and the biophysical cues that can drastically improve the reprogramming process have yet to be identified. Thus, future directions would be to demonstrate the clinical relevance of using biomaterials for the direct reprogramming of human cells and how the combination of biomaterials along with biochemical approaches can be used to facilitate the *in vivo* application of such strategies. Extensive studies have been performed using biomaterials in iPSC reprogramming, thus, these biophysical cues could also be adapted to study how they may influence the direct reprogramming paradigm. Importantly, the underlying mechanisms by which various biophysical factors influence direct reprogramming and modulate signaling pathways, epigenetic and/or chromatin states still await further investigation. These, together with robust characterization of temporal gene expression during the reprogramming course are crucial to direct the engineering of biomaterials that can mimic the induction of such gene expressions, which may further improve conversion efficiencies.

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- • Of special interest
- •• Of outstanding interest

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Figure 1. iPSC reprogramming versus direct cell reprogramming. Schematic diagram illustrating the process of differentiation, reprogramming and direct conversion. The traditional modes of delivery of the reprogramming factors used to manipulate cell fate are indicated. Recent studies have also unraveled a critical role played by biomaterials in modulating this process.

Figure 2. Influence of the microenvironment on cell fate and function. Cell behavior can be manipulated through altering biophysical and biochemical factors in the microenvironment.

Table 1 Summary of direct reprogramming strategies using biophysical cues

Initial cell population (Species*)	Target cell type	Reprogramming factors	Biophysical stimuli	*In vitro* / *In vivo*	References
TTF, Neonatal cardiac fibroblasts	Cardiomyocytes	*Gata4, Mef2c, Tbx5*	Mechanical stretch, substrate stiffness, microgrooves	*In vitro*	Sia et al, 2016
MEF	Neurons	*Ascl1, Brn2, Myt1l*	Microgratings, microposts	*In vitro*	Kulangara et al, 2014
MEF	Dopaminergic Neurons	*Ascl1, Pitx3, Nurr1, Lmx1a*	Micro- and nanogrooves	*In vitro*	Yoo et al, 2015
Skin fibroblasts (Hu)	Cardiac, neuronal, skeletal muscle	None	Radio electric conveyed fields	*In vitro*	Maioli et al, 2013

*Mouse unless stated otherwise

Hu: Human; MEF: Mouse embryonic fibroblasts; TTF: Tail tip fibroblasts

Figure

Figure 1

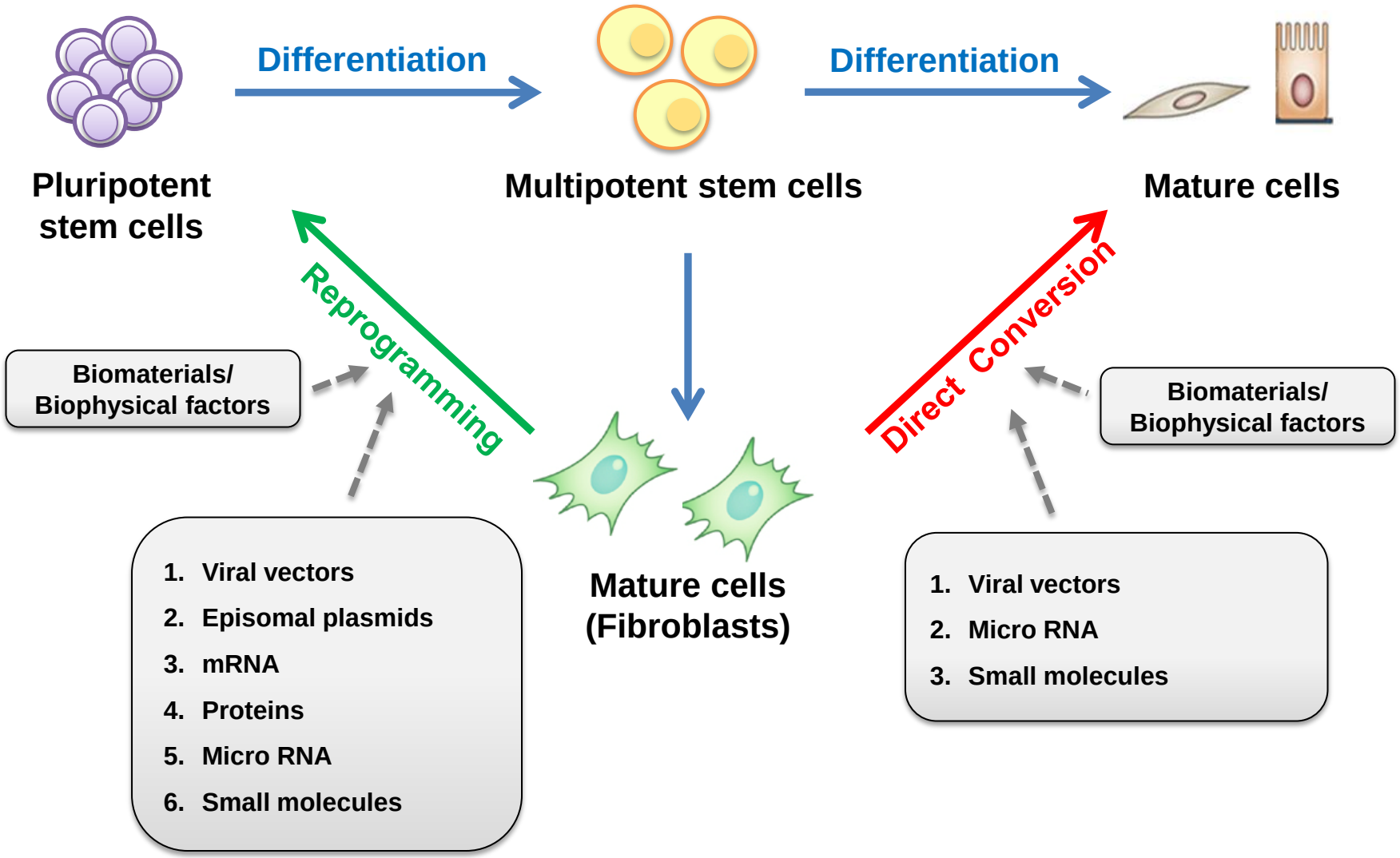
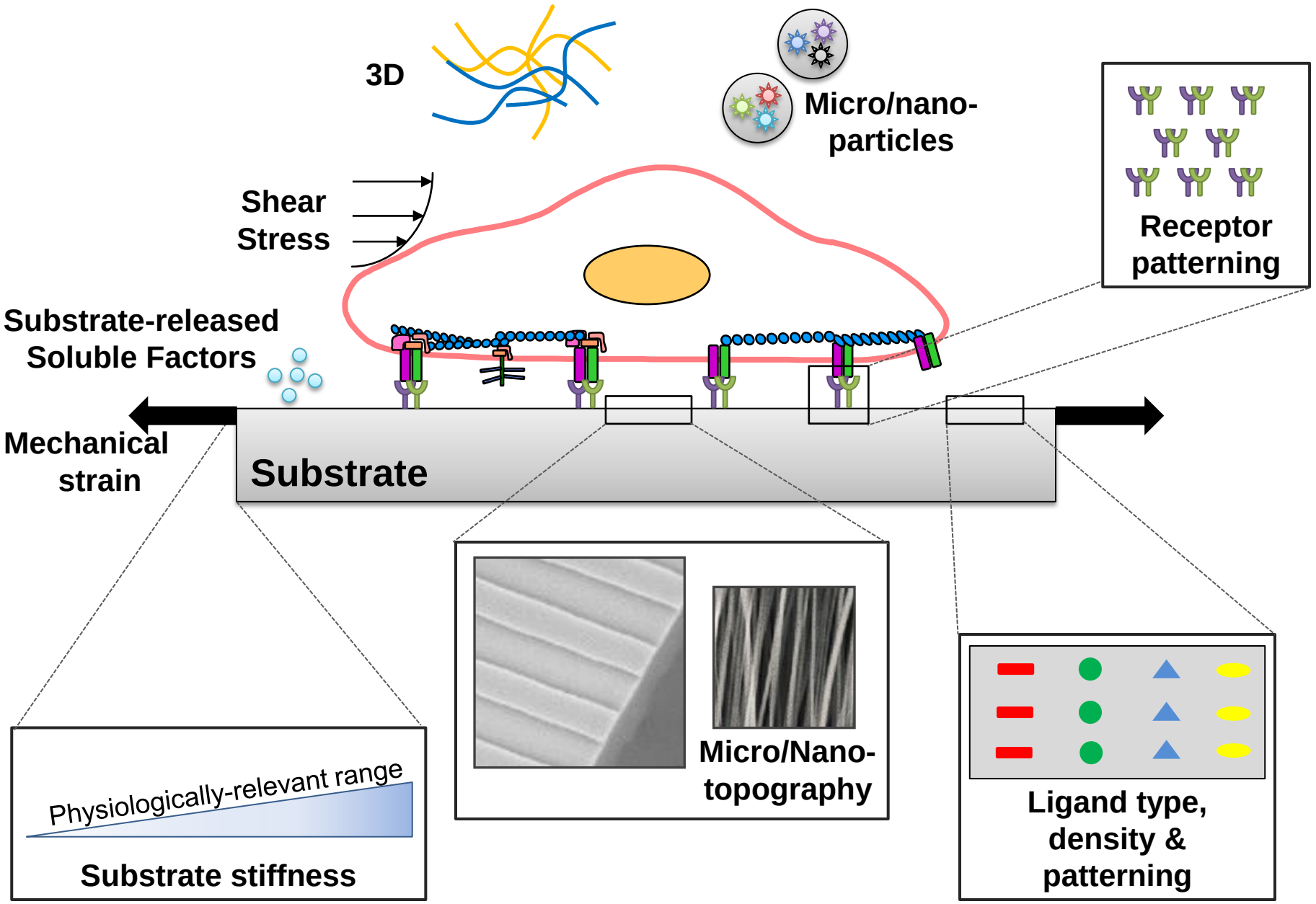


Figure 2


3D
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topography
Physiologically-relevant range
Substrate stiffness
Ligand type,
density &
patterning