Chapter

Spatiotemporal Regulation of Cell–Cell Adhesions

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Abstract

Cell–cell adhesions are fundamental in regulating multicellular behavior and lie at the center of many biological processes from embryoid development to cancer development. Therefore, controlling cell–cell adhesions is fundamental to gaining insight into these phenomena and gaining tools that would help in the bioartificial construction of tissues. For addressing biological questions as well as bottom-up tissue engineering the challenge is to have multiple cell types self-assemble in parallel and organize in a desired pattern from a mixture of different cell types. Ideally, different cell types should be triggered to self-assemble with different stimuli without interfering with the other and different types of cells should sort out in a multicellular mixture into separate clusters. In this chapter, we will summarize the developments in photoregulation cell–cell adhesions using non-neuronal optogenetics. Among the concepts, we will cover is the control of homophylic and heterophilic cell–cell adhesions, the independent control of two different types with blue or red light and the self-sorting of cells into distinct structures and the importance of cell–cell adhesion dynamics. These tools will give an overview of how the spatiotemporal regulation of cell–cell adhesion gives insight into their role and how tissues can be assembled from cells as the basic building block.

Keywords: optogenetics, cell–cell adhesion, differential adhesion hypothesis, reversible adhesion, subcellular resolution

1. Introduction

Cells adhere to the matrix and other cells around them, which fundamentally impacts their behavior. A thorough understanding of these adhesive interactions is also important to produce artificial tissues. Cell adhesions are formed by cell adhesion molecules on the cell surface such as integrins and cadherins which bind to the matrix and cadherins on neighboring cells, respectively [1]. These adhesion molecules transmit both physical and chemical signals between cells and their environment via the underlying cytoskeleton and intracellular signaling cascades [2].

1.1 Cell–cell adhesions

Cell–cell connections induce and receive biochemical signals and contractile forces from adjacent cells, and it is through theses stresses that cellular and tissue homeostasis is maintained [3]. The most abundant and well-studied cell–cell adhesion molecules are the cadherins. Cadherins such as E-cadherin, N-cadherin,

and P-cadherin, consist of five extracellular domains with a calcium-binding site between each domain (**Figure 1**). The cell–cell adhesion is initiated by the cadherins on adjacent cells forming homophilic interactions via the exchange of β-strands between the first extracellular domains [4] and from here the cadherin signal is transmitted into the cell via an intracellular tail domain. Force-dependent conformation changes in cadherins lead to the recruitment of binding partners such as α-catenin, β-catenin, and vinculin thereby conveying the chemical signal to the intracellular actomyosin network. These ensuing biomechanical and biochemical cascades direct scaffolding proteins toward cellular pathways regulating division, survival, structural morphologies [5, 6] epithelial-mesenchymal transition (EMT), cell-sorting, and collective cell migration [7].

1.2 Spatiotemporal regulation of cell–cell adhesions

Altering the number of cellular adhesions is critical to many biological processes during tissue development and cancer progression. For instance, the interconnected nature of epithelial cells, which line the surface of organs, tissues, and blood vessels, designates their polarity, which is critical to their function. EMT takes place when epithelial cells lose the adhesions to other cells and therefore their basal-apical polarity. The resulting mesenchymal cell has increased cellular motility and invasiveness. This process takes place naturally to produce the mesoderm, one of the germ layers, during embryonic development [8, 9], pro-inflammatory wound healing [10], and during cancer cell metastasis [11–13].

Before the development of the germ layers, the embryonic stem cells in the inner mass of the blastocyst are largely epithelial in characteristic; however, during germ layer development, gastrulation, the epithelial-like cells undergo EMT to form the mesoderm. I*n vitro* culturing of embryonic stem cells or epiblast cell colonies, shows that they lose expression of E-cadherin, vimentin, and N-cadherin, thus giving rise to cells with a mesenchymal phenotype. The opposite of EMT,

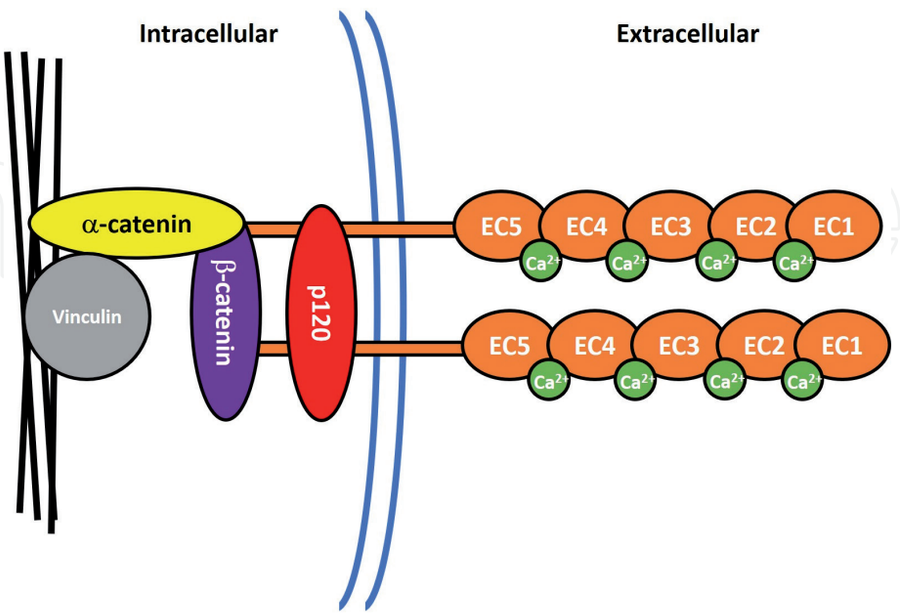


Figure 1.
E-cadherin dependent cell–cell adhesion. The E-cadherin consists of five extracellular domains, one transmembrane domain and an intracellular domain. During binding of two E-cadherin molecules the proteins p120, β-catenin, α-catenin, and vinculin get recruited to the intracellular domain leading to cytoskeletal adhesion and actomyosin based activation.

mesenchymal-epithelial transition (MET) also occurs naturally and can be seen in the procedure by which induced pluripotent stem cells are formed from fully differentiated cells. This process requires the transition from a cmesenchymal phenotype to an epithelial phenotype, and the activation of epithelial genes encoding epithelial cell junction proteins [8].

EMT extends to carcinomas as well, where a subpopulation of self-renewing cells, known as cancer stem cells, can efficiently generate new tumors. This can be seen in mammary carcinomas following the induction of EMT, which promotes the generation of clusters of invasive mammary gland cells [14]. The extent of these epithelial connections can also be seen in metastatic experiments involving the mammary cancer cell line MCF-7, which maintains an epithelial-like phenotype. In these experiments, MCF-7 is added on top of mammary endothelial cell sheets, and the invasiveness of MCF-7's was evaluated over increasing crossflow, it was revealed that the majority of MCF-7 cells could not form strong adhesions thereby failing to invade. Instead, the MCF-7 s remained rounded and rolled across the surface of the endothelial sheet [13].

Cadherin connections also guide cell migration through their intracellular connection to the cytoskeleton. For instance, in experiments examining the effect of cadherin adhesions in binary cell systems, it was revealed that single adhesions quickly recruit more cadherins to the initial contact site. Additionally, each recruited cadherin binds to the actin cytoskeleton preventing its depolymerization and enabling actomyosin-based mechanical signals [2, 15–17]. Additionally, cadherin-based stabilization of actin in migrating cells leads to *in situ* blebbing of the plasma membrane. These develop the leading edge for the cell, which in turn coordinates the migration of the cell [18]. In tissues with lots of interlocking cadherins, these effects lead to the development of leader cells, which migrate in front of the main body of follower cells. This is an event very common in angiogenesis, where sprouting endothelial cells lead to the development of new blood vessels [19].

1.3 Bottom-up tissue engineering

Another aspect for which controlled cell–cell adhesions are crucial is in bottom-up tissue engineering, in which single cells are organized into either planar or three-dimensional structures [20]. Since bottom-up engineering does not rely on external matrices to sequester the cells and instruct cellular arrangement the ability to spatiotemporally control the cell–cell connections is critical to building the desired structure. Techniques for creating bottom-up tissues include bioprinting, construction of cell sheets, and self-assembly of multicellular aggregates [20–23].

Self-assembled multicellular aggregates form by mixing multiple cell types such that microtissues with desired organization form. Generally, these structures form based on minimizing the potential internal energy resulting from cell–cell adhesions [24, 25]. Self-assembled aggregates have been used to construct multicell neuro-organoids comprised of cortical neural progenitor cells, endothelial cells, and mesenchymal stem cells. Different aggregates of each or a mix of two cells were first created in low-attachment 96-well plates. Following aggregate production, aggregates were then mixed to fuse the three cell populations. The resultant aggregate then sorted to form discrete layers within the aggregate. The cortical neural progenitor and endothelial cells developed into vascularized cortical brain tissue, while the mesenchymal stem cells took on a supportive role in the core of the aggregate [26]. With the ability to spatiotemporally control cell–cell adhesions it becomes possible to self-assemble cells together to produce more complex tissues that better recapitulate the *in vivo* structure.

1.4 Differential adhesion hypothesis

The cell sorting observed in tissues, self-assembled aggregates, and the developing embryoblast can be described by the differential adhesion hypothesis (DAH). The DAH explains cell sorting by comparing it to that of liquid mixtures, whereby the components (liquids or cells) arrange so that the internal free energy from cellular adhesions is reduced to a minimum to attain thermodynamic equilibrium [27–30]. Equilibrium is achieved via the active or passive motility of cells in the tissue rearranging with respect to each other to minimize stress and strain thereby limiting the internal energy [31]. Other aspects such as the cell's ability to round up to minimize their surface area, spreading of one cell over another, the fusion of two cellular aggregates, the sorting out behavior of mixed cell populations, and the hierarchy of the layering of two cell types further prove the analogy to liquid mixtures [31–33]. The DAH describes three different cases for multicellular assemblies in a mixture of two cell types (**Figure 2**) [30].

1.4.1 Intermixed

In this condition cells of type A and type B stay intermixed when the work of adhesion between the two cell populations (Wab) is higher than the work of cohesion of a single cell type (Wa and Wb) as this results in the maximal adhesion.

1.4.2 Enveloped

An enveloped arrangement of cells, occurs when one cell type is in the center and the secondary at its periphery. This arrangement forms when the average work of cohesion of cell type A and cell type B is greater than the work of adhesion

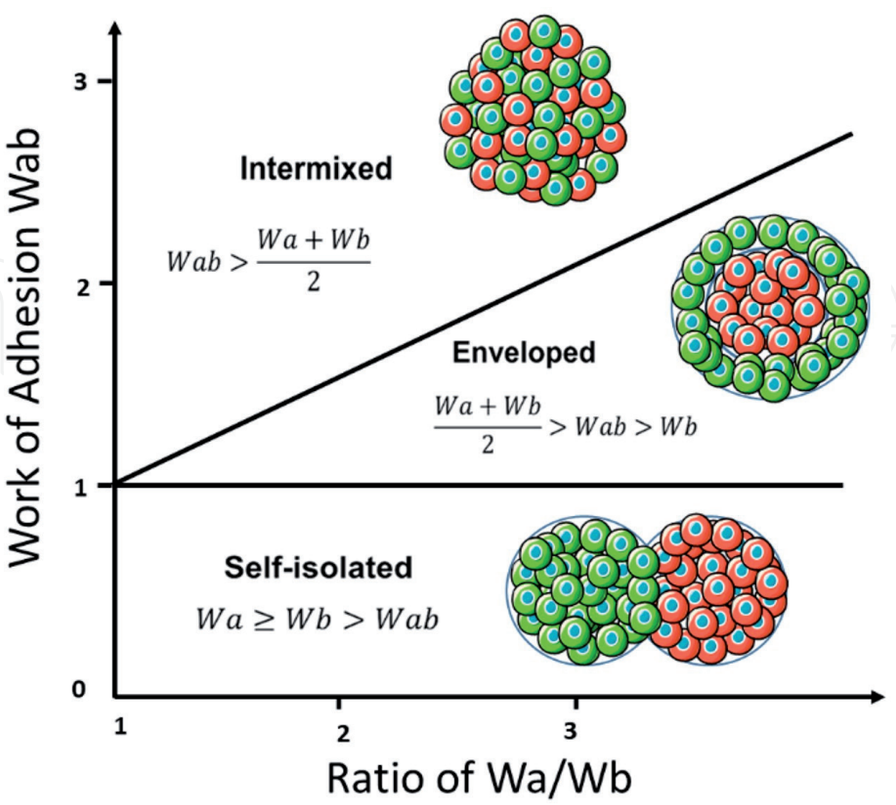


Figure 2.
Differential adhesion hypothesis (DAH). Different cell assemblies form at equilibrium depending on the work of adhesion between cells of type a (W_a), cells of type b (W_b) and cells of type a and type b (W_{ab}).

between the two cell types and the work of cohesion of one cell type is smaller than the work of adhesion between the cell types. Herein, the cell type with the stronger cohesion, type A, forms the core and the less cohesive cell type, type B, surrounds this core.

1.4.3 Self-isolated

In a self-isolated system the two cell types form separate assemblies because the work of adhesion between the cell types is smaller than the work of cohesion within either population. In this case each cell type will self-isolate with no intermixing.

Numerous studies with cells expressing different types and amounts of cadherins have demonstrated these sorting schemes [34–36]. These studies show that the differences in homophylic and heterophilic cell–cell adhesions determine the outcome and the origin of these differences on adhesions are not important for the result.

2. Possible ways of controlling cell–cell adhesions

Currently, there are only a few tools for controlling cell–cell adhesion, which enable the studying of the underlying biology and for bottom-up tissue engineering. Important aspects to consider in the control of cell–cell adhesions are their specificity, their dynamics, and most importantly, their spatiotemporal regulation. The current approaches can be divided into two; the modification of the cell surface with chemically reactive groups and the genetic modification of cells to alter the expression of cell adhesion molecules [37].

In the following sections, we will discuss options of regulating cell–cell adhesions using reactive chemical groups and then consider photoregulation of cell–cell adhesions using light-responsive small molecules and finally optogenetic approaches. Light is especially advantageous as a trigger for cell–cell adhesions since light, as opposed to other stimuli like chemical inputs, temperature, redox etc., can be delivered with superior spatial and temporal control. Using a focused beam of light enables precise subcellular delivery, which can exclude the surrounding area. Secondly, light allows for temporal control as it can be turned on or off instantly making delivery or removal at the desired point instantaneous [38, 39].

2.1 Introduction of reactive groups to induce cell–cell adhesions

A general strategy for initiating user-controlled cell–cell interactions is to introduce reactive chemical groups on the cell surface. These chemical groups are not genetically coded and thus do not require genetic engineering to add them to the surface. Such chemical groups are introduced through the fusion of lipid vesicles containing the chemical reactive groups or through metabolic labeling with non-natural sugars bearing bioorthogonal functional groups with the cell [40]. For instance, complementarily reactive ketone and oxyamine groups or alkyne and azide groups can be introduced on the plasma membrane of cells [41]. Consequently, when cells with complementary reactive groups are mixed, the functional groups on the cell surfaces react and cells are connected through covalent bonds [42, 43]. In general, so-called click reactions, that take place in water, do not form toxic side products and do not interfere with other functional groups found in biomolecules. Alternatively, noncovalent interactions with high specificity can be used to form cell–cell adhesions. For this purpose, the binding of biotin to streptavidin [44–46] or the hybridization of complementary single-stranded DNA [47–49] is

employed. DNA-based cell–cell adhesions open the possibility to form diverse structures with varying cell types and cellular connectivity owing to the high specificity of these interactions; however, DNA adhesions show limited reversibility making migratory sorting impossible, and covalent and strong noncovalent links between cells permanently glue them together [50].

2.2 Spatiotemporal control over cell–cell adhesions using light responsive small molecules

Light sensitive small molecules, such as nitrobenzenes and azobenzenes, have been used to control cell–cell adhesions in space and time. For example, light cleavable nitrobenzene groups can be introduced to oxyamine linkers at the cell surface. When this cell population is mixed with a second population of cells with a ketone group at the cell surface multicellular clusters formed. These cell cluster can then be broken up into single cells upon UV-light illumination since UV-light cleaves the nitrobenzyl moiety [51]. Such a photocleavable linker only allows for a single reversion of the cell–cell adhesions. To achieve cell–cell adhesions that can be switched on and off repeatedly a linker with a photoswitchable azobenzene group was developed. β-cyclodextrins can be clicked onto the surfaces of cells and when a divalent photoswitchable azobenzene (azo) linker (azo-PEG-azo) is added in the dark the cells will link together. This is because, in the dark, the trans configuration of the azobenzenes binds to the cyclodextrin moieties linking the cells together. Upon UV illumination the azobenzene switch to the cis conformation, which results in the release from the cyclodextrin and the dissociation of the cell–cell interactions. The azobenzene can then be switched back to the trans configuration with blue light illumination, thus allowing for the formation of new cell–cell adhesions [52]. These studies represent great advances in the field and allow for spatiotemporal control over cell–cell adhesions. However, the use of UV-light is damaging to DNA and therefore to cells, and secondly, the chemical modifications cannot be maintained over long periods of time. Thus, a system which utilizes biocompatible light and can be expressed over long times would be more beneficial to bottom-up tissue engineering since cell proliferation is a key component of any built tissue. For this purpose, a genetically engineered system, which allows for propagating the modification at the cell surface would be desirable.

2.3 Optogenetic control of cell–cell interactions

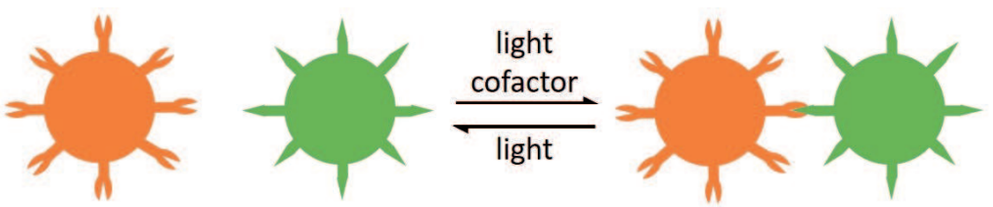
Cell–cell adhesions can be photoregulated by expressing bioartificial light-responsive proteins on the surfaces of cells as adhesion receptors. Numerous light-responsive proteins from algae, plants, bacteria, and engineered proteins change their conformation upon light illumination and bind to other proteins in a light-dependent manner through non-covalent protein–protein interactions [53–56]. In these optogenetic approaches, complementary light-dependent binding partners are expressed in the surfaces of different cell types by transfecting these proteins along with a plasma membrane localization sequence and a membrane anchoring sequence. Following translation, the localization sequence ensures that the protein is exported to the cell membrane where the extracellular portion operates as a bioartificial cell adhesion receptor [51, 52, 57]. For instance, the proteins Cryptochrome 2 (CRY2) from *Arabidopsis thaliana* and its blue light-dependent binding partner cryptochrome-interacting basic helix–loop–helix (CIBN) protein, were expressed on the surfaces of MDA-MB-231 cells, which do no form native cell–cell adhesion. When cells expressing CRY2 and CIBN at their surface are mixed and

cultured in the dark, no cell–cell adhesions form similar to the parent MDA-MB-231 cell line. However, if these cells are cultured under blue light, the cells grow in clusters indicating the formation of cell–cell adhesions (**Figure 3**). Moreover, the cell–cell interactions formed under blue light can be reversed in the dark, allowing for repeated deconstruction and reconstruction with light-dependent control [58]. This optogenetic approach has the advantage that the cell–cell adhesions can be triggered with visible blue light, which is non-toxic to the cells and the cell surface modifications are passed on to daughter cells following cell splitting.

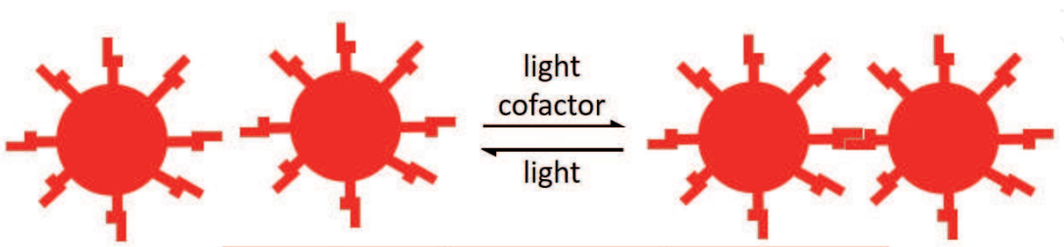
The large repertoire of photoswitchable protein–protein interactions allows for the formation of bioartificial cell–cell adhesions with different properties in terms of cell–cell adhesion mode, the light of color the adhesions responds to, reversion kinetics in the dark, and cell–cell adhesion dynamics [53–55].

In biology, cells can either interact with cells of their own type forming homophilic interactions or cells of another type forming heterophilic interactions.

To obtain light-responsive homophilic cell–cell adhesion, proteins that homodimerize under light are used as a mediator of cell–cell adhesion. For this purpose, the proteins Vivid, a member of the light oxygen voltage (LOV) domain from *Neurospora crassa*, and cyanobacterial phytochrome 1 (Cph1) from *Synechocysitis sp. PCC 6803* were used as these proteins homodimerize under blue and red light, respectively (**Figure 4**). Cells expressing Vivid at their plasma membrane form cell–cell adhesion exclusively when illuminated with blue light but not with red light. The reverse is true for cells expressing Cph1 at their cell surface, which only



Heterophilic	Partner	On	Off
iLID	Nano/SspB	Blue	Dark
pMag/pMagHigh	nMag/nMagHigh	Blue	Dark
CRY2	CIB1/CIBN	Blue	Dark



Homophilic	On	Off
Vivid	Blue	Dark
CarH	Dark	Green-Blue
Cph1	Red	Far-Red

Figure 3.
Optogenetic proteins bind either in hetero or homophilic complexes. In heterophilic optogenetic systems an optogenetic protein undergoes conformational changes that enable the binding to a target protein. Homophilic optogenetic proteins also undergo conformation changes, but here a homomer is formed. iLID (improved light induced dimer), CRY2 (Cryptochrome 2), CIB1/N (cryptochrome-interacting basic helix–loop–helix/truncated), Cph1 (cyanobacterial phytochrome 1).

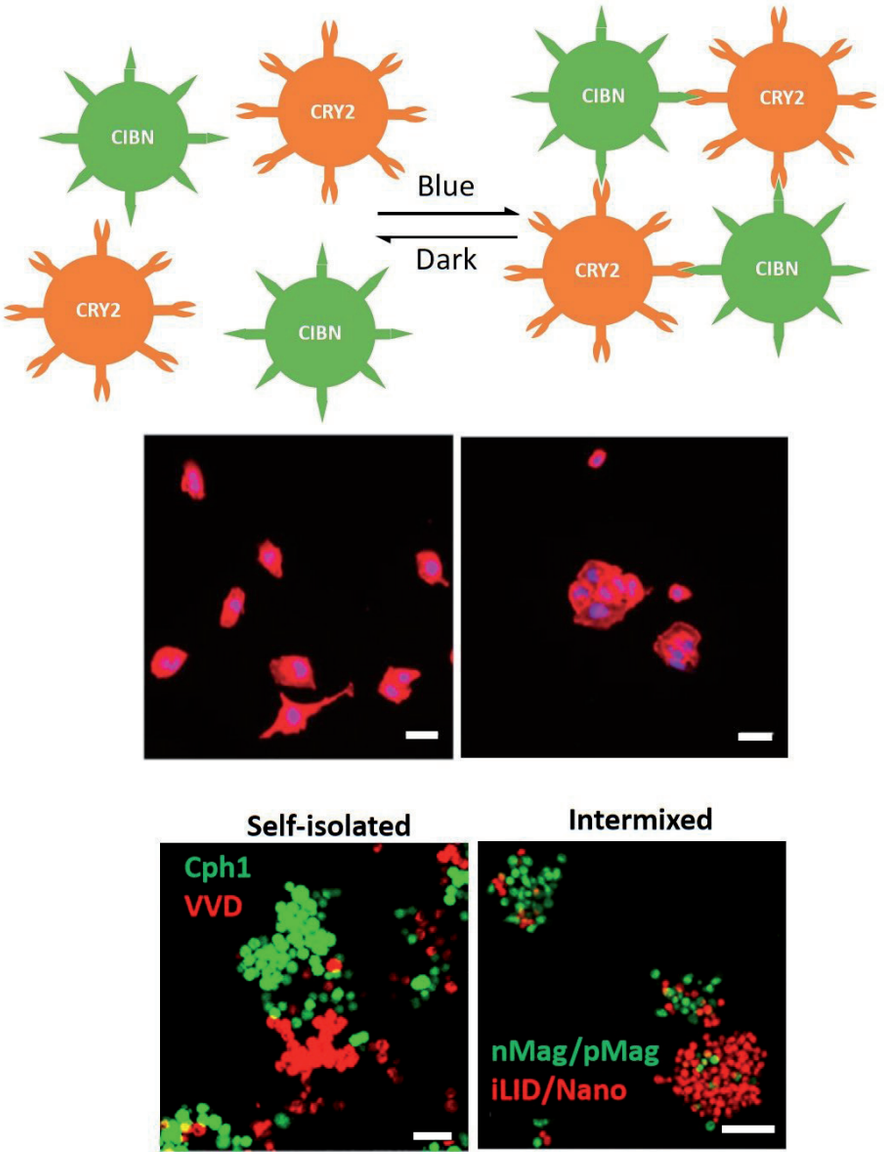


Figure 4.
Co-culture of optogenetic proteins results in cluster segregation. When colloidial particles are labled with the iLID/Nano, nMag/pMag, or nMagHigh/pMagHigh clusters of particles can be seen to form with respect to the kinetics of the system (adapted from Müller et al. [62]). In cellular systems utilizing the vivid (VVD) and Cph1 systems descrete clusters are observed rather than any intermixing (adapted from Rasoulinejad et al. [57]).

form cell–cell interactions under red light and not in the dark or under blue light. Similarly, the blue-green light-responsive protein, CarH from *Thermus thermophilus*, has been used to mediate homophilic cell–cell interactions. The formation of a CarH homotetramer allows it to form cell–cell adhesions between cells expressing CarH on their surface in the dark [59]. The CarH tetramer irreversibly degrades when exposed to blue-green light and hence the CarH based cell–cell adhesion can only be reversed once [59, 60].

Light responsive heterophilic cell–cell adhesions, can be achieved by proteins that heterodimerize under light to form cell–cell adhesions. For this purpose, different heterodimerization pairs that form under blue light and reverse in the dark were used. These include the binding of the improved light-induced dimer (iLID) to Nano [61], the binding of the Vivid-based proteins nMag and pMag and the previously-described binding of CRY2 to CIBN. These different protein pairs provide different interaction strengths, reversion kinetics in the dark, and protein–protein dynamics.

2.4 Cell–cell adhesion dynamics dictate the structure of multicellular assemblies

The assembly of multicellular structures does not just depend on the strength of the underlying cell–cell adhesions but also their dynamics. If cell–cell adhesions are dynamic, meaning that formed protein–protein interactions constantly form and disassemble within the chemical equilibrium, cells can move with respect to each other and maximize the number of adhesive contacts they form. This scenario is observed in mixtures of iLID and Nano expressing cells, which assembled into spherical and compact clusters. If cell–cell adhesions are not dynamic, meaning that once protein–protein interactions form that they do not reverse, cells stick to the first cell they meet and cannot move to areas with potential higher numbers of adhesions. For example, mixtures of nMagHigh and pMagHigh or nMag and pMag expressing cells assemble into ramified branched structures, which are kinetically trapped. Optogenetics allows for the altering of the dynamics of the cell–cell adhesion by turning light on and off. The ramified structures formed with nMag and pMag cells could then be converted into compact spheres under pulsed illumination (5 min on, 5 min off), allowing the adhesions to dissipate and the cells to move.

2.5 Regulation of cell sorting using photoswitchable cell–cell adhesions

Different types of photoswitchable cell–cell adhesions can be mixed to obtain cell sorting within multicellular mixtures and organize cells as predicted by the DAH. For example, when cells expressing Vivid or Cph1 at their cell surface were mixed and illuminated with either blue or red-light clusters of cells with Vivid or Cph1 cells formed, respectively. When both blue and red light was used self-isolated clusters containing either Vivid or Cph1 cells were observed (**Figure 4**) [57]. That means that the adhesive force for Vivid and Cph1 is lower than that for the homodimers formed for each system due to the specific protein–protein interactions. Similarly, also different pairs of heterophilic cell–cell adhesions can be used to achieve self-sorting in mixtures containing four different cell types. In mixtures of iLID, Nano, nMag, and pMag expressing cells, two types of multicellular aggregates assembled each containing one of the protein pairs (iLID/Nano or nMag/pMag) [62]. It should be noted that cell sorting is only possible if the system is under thermodynamic control and is not observed if kinetically trapped structures form. Therefore, mixtures of iLID, Nano, nMagHigh and pMagHigh do not sort into distinct clusters.

2.6 Photoswitchable cell–cell adhesions controlling cellular function

Cell–cell adhesions play an important role in many cellular functions, and the adhesions resulting from the optogenetic proteins are no different. Using CarH based homophilic cell–cell adhesions, the spatiotemporal control of migrations was assessed by measuring the rate and the morphology of cells migrating during a wound-healing assay. The spatiotemporal element was carried out by illuminating discrete sections to depolymerize the cell–cell adhesion. Cells with intact CarH adhesions in the dark showed significantly enhanced migratory potential compared to cells illuminated with green light, which dissociate the cell–cell adhesions. This was characterized by cells remaining together and thus migrating as a single cell wall resulting in faster migration. Cells that were illuminated with blue-green light broke away from the migratory front and engaged in random walking resulting in a slower overall migration rate [59].

Additionally, spatiotemporal control of the cell–cell adhesion complex has been shown in experiments where the β-catenin binding domains on E-cadherin and α-catenin have been replaced with the Halo and SNAP tags, respectively. The Halo/SNAP system incorporates the UV-light photocleavable small molecule Ha-pl-BG, so adhesions can be reversed upon UV illumination. This system was then applied to MDA-MB-468, which do not express endogenous E-cadherin to assess the efficacy of the system. Using the system cell–cell adhesions could only be observed when the cofactor was present and were degraded rapidly under UV-light. To illustrate the spatiotemporal control, A431 cells, with knocked out α-catenin, were labeled with the Halo/SNAP system and cultured overnight to initiate connections between cells. Specific adhesions between cells were then targeted and illuminated with UV-light. Only the targeted connections were degraded leaving the other connections intact.

3. Conclusion

The spatiotemporal nature of cadherin-based cell–cell adhesions enables cells to self-sort, assemble into tissues, or can lead to cellular differentiation. However, these adhesions cannot be exogenously controlled, and as such make the construction of bottom-up tissues difficult to manage. Chemical means for binding cell membranes together are too rigid and offer limited reversibility. There is also a lack of spatiotemporal control. However, light is non-invasive, highly biocompatible, and can be delivered in a spatiotemporal fashion. Through the delivery of optogenetic proteins to the cell membrane, the construction of spatiotemporal cell–cell adhesions has been achieved. These proteins can respond to a wide range of wavelengths enabling the use of multiple pairs to construct larger structures, form reversible adhesions, and offer superior kinetics to other adhesion methods.

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Conflict of interest

The authors have no conflicts to disclose.

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