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MDCK cyst formation in *3-D Life* Hydrogels

1. Introductory Notes

- The *3-D Life* Hydrogel technology and its applications are described in the *3-D Life* Hydrogels User Guide which can be downloaded on the Cellendes web page: www.cellendes.com/pdfs/manual_hydrogel.pdf.
- For the generation of MDCK cysts, cells are embedded in hydrogels formed by crosslinking of dextran or polyvinyl alcohol (PVA) polymers with polyethylene glycol (PEG). Compared to dextran-based gels, PVA-based gels are more stable during long time incubation periods of more than 2 weeks. Unlike PVA-based gels, dextran-based gels can be degraded with dextranase which allows the isolation of cells from the gel after culture for downstream applications.
- The formation of cysts can be visualized via fluorescent staining of the actin cytoskeleton and nuclei (see Figure 1 below).
- In the protocol, both maleimide-functionalized dextran (Mal-Dextran) and maleimide-functionalized PVA (Mal-PVA) is referred to as Mal-Polymer.
- Prior to crosslinking of Mal-Polymers with thiol-functionalized PEG (PEG-Link), the respective Mal-Polymer is modified with RGD Peptide.

2. Embed cells in hydrogel

Reagents and materials:

***3-D Life* products:**

3-D Life Dextran-PEG Hydrogel Kit (Cat # G90-1) or *3-D Life* PVA-PEG Hydrogel Kit (Cat # 09-G-001)

3-D Life RGD Peptide (Cat # 09-P-001)

Reagents and materials not included in the *3-D Life* product:

Cell culture plate: e.g. 8-chamber slide or multiwell plate, preferably with glass bottom or equivalent for inverse microscopy.

Cell culture medium: DMEM, 10% fetal calf serum, 2 mM glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin

Sterile PBS w/o Ca/Mg (PBS$^-$)

MDCK cells: cell suspension in PBS$^-$ at a cell density of 2.5x 10^6 cells/ml

Other material: Eppendorf tubes, pipet tips, micropipets, serological pipets, ice

Preparations:

- If hydrogel reagents are provided in lyophilized form, dissolve the lyophilisates according to the instructions in the accompanying Data Sheets. If hydrogel reagents are provided frozen, thaw all reagents at room temperature. Make sure that salts in the 10x CB pH 5.5

are completely dissolved. Do not put 10x CB pH 5.5 on ice: this may cause the salts to crystallize.

- Place Mal-Polymer on ice.

Note: Do not expose *3-D Life* thiol-containing reagents (RGD Peptide, Thioglycerol, PEG-Link) to air longer than necessary to avoid oxidation of the thiol-groups. Close cap after each use.

Experimental procedure:

The volumes of gel components for 30 µl gels are listed in Table 1. If several gels with the same composition are generated, a reagent mix (Reagent Mix A) consisting of 10x CB, Water, Mal-Polymer, RGD Peptide and the cell suspension can be prepared in multiples of the indicated volumes (see 3x example in Table 1).

Note: We recommend preparing the reagent mix with one excess volume of each component to avoid a potential shortage of reagent mix in case slight inaccuracies in pipetting occur.

Table 1: *Reagent volumes for 30 µl gels crosslinked with 3 mmol/l maleimide- and SH groups, and modified with 0.5 mmol/l RGD Peptide.*

3-D Life reagents	Final concentrations in the gel	1x (µl)	3x (µl)	
10x CB, pH 5.5	n.a.	2.4	7.2	= Reagent mix A
Water	n.a.	12.8	38.4	
Mal-Dextran or Mal-PVA (30 mmol/l maleimide groups)	3.5 mmol/l maleimide groups	3.5	10.5	
RGD-Peptide (20 mmol/l SH groups)	0.5 mmol/l SH groups	0.8	2.4	
Cell suspension (2.5x 10^6 cells/ml)	1.5x 10^4 cells	6.0	18.0	
PEG-Link (20 mmol/l SH groups)	3 mmol/l SH groups	4.5	3x 4.5	
Total		30.0	3x 30.0	

Note: The volumes of gel components of any gel composition can be easily determined with the online calculator on www.cellendes.com/calculation_form.php.

All steps below are performed under a sterile hood:

- Combine Water, 10x CB (pH 5.5) and Mal-Polymer in an Eppendorf tube. Mix well.
- Add the RGD Peptide and mix immediately to ensure homogenous modification of the Mal-Polymer with the peptide.
- Incubate sample for 5-10 min at room temperature to allow for the RGD Peptide to attach to the maleimide groups of the polymer.
- Place 4.5 µl of crosslinker (PEG-Link) on the surface of a culture dish.

- Add cells to the reaction tube containing the peptide-polymer conjugate to complete Reagent mix A.
- In a pipet tip, transfer 25.5 µl of Reagent mix A to the 4.5 µl PEG-Link on the dish and quickly mix both by pipetting up and down three times. Avoid the formation of air bubbles. Leave the mix on the surface of the culture dish. Wait for approximately 3 minutes to let the gel form.

  Note: Gel formation starts after a few seconds of mixing. Complete mixing step as fast as possible to avoid gel formation in the pipet tip.
- Optional: test gel formation by careful inspection with a pipet tip.
- Once the gel has formed, add cell culture medium until the gel is covered.
- Use lid to cover the culture dish, then place the dish in the incubator.
- Renew the medium after 1 hour.
- Replace medium every 2-3 days.

Instead of the RGD-Peptide, Thioglycerol can be added as described above. In this case the gel does not provide cell attachment sites and can be used as a control to RGD Peptide-modified gels. Cellendes also offers a scrambled version of RGD Peptide for control experiments (*3-D Life* Scrambled Peptide, Cat # 09-P-003).

3. Fluorescent staining of the actin cytoskeleton and nuclei

Reagents and materials (not included in the *3-D Life* product):

Wash buffer: PBS^-, PBS^+ (+ Ca/Mg)

Fixative: 4% paraformaldehyde (pFA) in PBS^+

Detergent: 0.1%Triton X-100 in PBS^-

Actin staining: Phalloidin-TRITC (e.g. Sigma #1951); 0.5 mg/ml stock solution in methanol. Final staining concentration: Dilute stock solution 1:200 in PBS^-.

Nuclear staining: Syto 24 Green (Invitrogen #S7559); 50 µmol/l stock solution in PBS^-. Final staining concentration: Dilute stock solution 1:300 in PBS^-.

Experimental procedure:

Perform all steps that include fixative under a fume hood! In all washing and incubation steps use volumes of solutions large enough to completely cover the hydrogels.

- Remove medium and wash gels once with PBS^+.
- Add fixative. Incubate for 30-60 min at room temperature.
- Remove fixative and wash gels 3x 5 min with PBS^-.
- Add Detergent and incubate for 10 min.
- Wash gels 3x 5 min with PBS^-.
- Add Phalloidin-TRITC and incubate for 1h in the dark.
- Wash gels 3x 5 min with PBS^-.

- Add Syto 24 Green and incubate for 20 min in the dark.
- Wash gels 3x 5 min with PBS^-.
- Keep gels immersed in PBS^-. Gels can be stored at 4°C up to several weeks. After storing for more than one day, the staining may become diffuse due to dislocation of staining reagents from their target molecules. To avoid evaporation when stored for more than one day seal the dish with parafilm.
- Cells can be visualized by fluorescence microscopy. Cysts form only in the presence of RGD-Peptide (compare A and B in Figure 1).

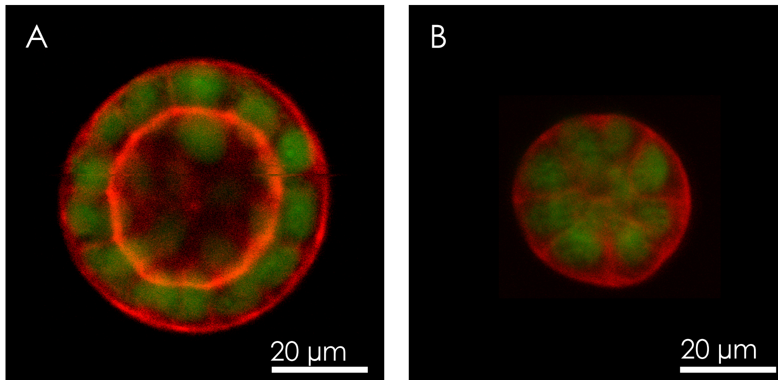

Figure 1: Confocal laser-scanning microscopy of MDCK cell aggregates after 14 days of incubation in PVA-PEG Hydrogel modified with 1 mmol/l RGD Peptide (A) or Thioglycerol (B), respectively. Red: Actin. Green: nuclei.

Note: This result is also obtained by using the Dextran-PEG Hydrogel and 0.5 mmol/l RGD Peptide as described above.