

HERLYN LAB CELL CULTURE TECHNIQUES

- 1. Melanocyte Growth & Isolation**
- 2. Skin Reconstruction**
- 3. Melanoblast Media**
- 4. Melanoma Isolation Media**
- 5. Vessel Reconstructs**
- 6. Melanoma Stem Cell Media**

1. MELANOCYTE GROWTH AND ISOLATION

Day 1

1. Prepare the following in a laminar flow hood: one pair of sterile forceps, curved scissors, and surgical scalpel blade; 5 ml of dispase II in a sterile centrifuge tube; 20 ml of Ca^{2+} , Mg^{2+} - free HBSS in a sterile non-tissue culture Petri dish; and 10 ml of 70% ethanol in a separate Petri dish.
2. Soak the skin specimens in 70% ethanol for 30 seconds. Transfer skin to another Petri dish containing HBSS to rinse off ethanol (see notes 1 and 2).
3. Cut skin-ring open and trim off fat and subcutaneous tissue with scissors (see note 3).
4. Cut skin into pieces (approximately $5 \times 5 \text{ mm}^2$) using the surgical scalpel blade with one-motion cuts (see note 4).
5. Transfer the pieces into the tube containing dispase II. Cap, invert, and incubate the tube in the refrigerator at 4°C for 15-18 hours.

Day 2

1. Prepare the following in a laminar flow hood: one pair of sterile forceps and a surgical scalpel blade; 10 ml of Ca^{2+} , Mg^{2+} -free HBSS in a sterile, non-tissue culture Petri dish; two empty sterile Petri dishes; 2 ml of 0.5% trypsin/versene solution in a 15-ml centrifuge tube; and 8 ml of soybean trypsin inhibitor.
2. Pour tissues in dispase II into one of the empty Petri dishes. Transfer tissue pieces to the Petri dish. Separate epidermis (thin translucent layer) from dermis (thick opaque layer) using

the forceps and scalpel blade. Hold the dermal part of the skin piece with the forceps, and gently slide the epidermal side off with a scalpel blade. Discard the dermis immediately (see note 5).

3. Add a drop of Ca^{2+} , Mg^{2+} -free HBSS on the resulting epidermis to avoid drying while isolating the epidermis from the remaining skin pieces. Repeat the procedure in step 2 on each piece of tissue. Mince the epidermal sheets as small as possible using a surgical scalpel blade.
4. Transfer the collected epidermis from the Petri dish to a centrifuge tube containing 0.5% trypsin/versene solution. Incubate the tube at 37°C for 3-5 minutes depending on cell disaggregation. Pipette up and down vigorously 80x with a 5 ml pipette to release single cells from epidermal sheets. Neutralize trypsin with soybean trypsin inhibitor. Centrifuge for 5 minutes at 1,200 rpm at room temperature. Aspirate the supernatant which may contain remaining stratum corneum. Resuspend the pellet with Melanoblast Media.
5. Place the resulting epidermal cell suspension at approximately concentration 2×10^5 cells/cm² in the tissue culture vessel. Incubate at 37°C 5% CO₂ for 48-72 hours.

After 2 Days

1. Change media to new Melanoblast Media to remove non-adherent cells. Medium change should be performed twice each week. If the culture is contaminated with fibroblasts, start treatment with MGM containing 100 ug/ml of geneticin (G418) for 2-3 days. Seventy percent confluent primary cultures can be obtained in 2 weeks.
2. **Subcultivation.** Primary cultures established from foreskins usually reach 70% confluence within 12 days. Cultures are washed with Ca^{2+} , Mg^{2+} -free HBSS and treated with trypsin-versene solution (90 ml versene + D-glucose and 10 ml 2.5% trypsin) at room temperature for 1 minute, harvested with Leibovitz's L-15 containing 10% heat-inactivated FCS, centrifuged at 1,200 rpm for 5 minutes, resuspended in Melanoblast Media, reinoculated at 10^4 cells/cm², and serially passaged. Medium is changed twice each week.
3. **Cryopreservation.** Melanocyte suspensions harvested by trypsin-versene and Leibovitz's L-15 containing 10% FCS are centrifuged at 1,200 rpm for 5 minutes and resuspended in heat-inactivated FCS containing 5% DMSO as a cryopreservative. Cells are normally suspended at a density of 10^6 /ml and transferred to cryotubes. The tubes are then placed in a plastic sandwich box that is immediately transferred to a -70°C freezer. The insulation of the box ensures gradual cooling of the cryotubes and results in over 80% viability of the cells upon thawing. After overnight storage in the freezer, the cryotubes are placed in permanent storage in liquid nitrogen.
4. **Thawing.** The melanocyte suspension is thawed by placing the cryotube in a water bath at 37°C . When the cell-preservative medium is almost but not totally defrosted, the outside of the tube is wiped with 70% ethanol. The cell suspension is then withdrawn, quickly diluted in

Leibovitz's L-15 containing 10% FCS at room temperature, centrifuged, and resuspended in fresh Melanoblast Media. Cell viability is determined by trypan blue exclusion. The resulting melanocytes are then seeded at a density of $10^4/\text{cm}^2$.

NOTES:

- 1. Tissue Source and Collection:** The sources of tissue for melanocyte cultures are human neonatal foreskins obtained from routine circumcision and normal adult skin acquired from reduction mammoplasty. At the time of excision, the skin is placed into a sterile container with 20 ml of normal skin transporting medium (2 ml penicillin/streptomycin + 2 ml gentamycin in 1L Ca^{2+} , Mg^{2+} -free HBSS) supplied in advance and kept near the surgical area at 4°C . Specimens are delivered immediately to the tissue culture laboratory or stored at 4°C . Neonatal foreskins can be kept for up to 48 hours and normal adult skin, for up to 24 hours. However, the fresher the specimens, the higher the yield of live cells upon isolation.
- 2. Sterilization of Skin Specimens:** Reduce contamination by a short treatment (30 seconds) of intact skin with 70% ethanol in a laminar flow hood. After sterilization, rinse samples with HBSS.
- 3. Preliminary Tissue Preparation:** Place tissues in a 100-mm non-tissue culture Petri dish and remove most of the subcutaneous fat and membranous material with curved scissors.
- 4. Adjustment of Tissue Size for Trypsinization:** To improve reagent penetration, cut the skin samples into small pieces (approximately $5 \times 5 \text{ mm}^2$) rinsed in HBSS.
- 5. Separation of Epidermis from Dermis:** Dispase II splits epidermis from the dermis along the basement membrane. Since melanocytes are located just above the basement membrane, removal of this lowest layer of epidermal cells requires some effort. Each piece of skin is held with forceps with dermal side down. The epidermal sheet is detached by sliding it off with a scalpel blade. To prevent the epidermis from drying, a drop of Ca^{2+} , Mg^{2+} -free HBSS can be added to the resulting epidermal sheet. To avoid potential sources of fibroblast contamination, dermal pieces should be discarded immediately once they are separated from the epidermis, and the forceps used to hold the dermis should never touch the epidermal sheets. Contaminated dermis is easily recognized by its white color in contrast to the yellowish-brown color of the epidermis. Isolated epidermal sheets in cell dispersal solution are then transferred to a centrifuge tube.

2. SKIN RECONSTRUCTION

For a video on how to generate a 3D human skin reconstruct for studies of melanocyte, melanoma and skin cell biology, please [click here](#). This video was generated in the Herlyn laboratory as part of [a publication](#) in the Journal of Visualized Experiments.

Dermis

For dermal reconstruction, 1 ml of a cell-free buffered collagen solution consisting of bovine collagen, type I at a final concentration of 0.78-1.01 mg/ml in Minimal Essential Medium with Earle's Salts, 200 mM L-glutamine, FCS, and 7.5% sodium bicarbonate was added to tissue reconstruct trays (Fig. 1). This pre-coated acellular layer was then overlaid with 3 ml of fibroblast-containing collagen (2.4×10^4 /ml). After 4-7 days of incubation at 37°C, the fibroblasts had contracted the collagen gel, which formed a concave central area for subsequent seeding of epidermal cells.

Epidermis

For epidermal reconstruction, the mature dermal reconstruct was rinsed and equilibrated with 14 ml Ca^{2+} , Mg^{2+} -free HBSS containing 1% dialyzed FCS (12 ml outside and 2 ml on top of the polycarbonate insert). After 1 hour, HBSS + 1% dFCS was removed, and replaced with Melanocyte Reconstruct Media (MRM) (1.1) (12 ml and 1.7 ml outside and inside of the insert, respectively). Neonatal foreskin melanocytes and keratinocytes in a ratio of 1:5 (500,000 cells total/reconstruct) were dispensed on top in a total volume of 200 μl MRM. Seeded melanocytes and keratinocytes were allowed to attach and proliferate. After 4 days of submerged cultures, with a feeding after 2 days, skin reconstructs were lifted to the air-liquid interface and medium was switched to maintenance medium with omitted EGF and increased CaCl_2 concentration.

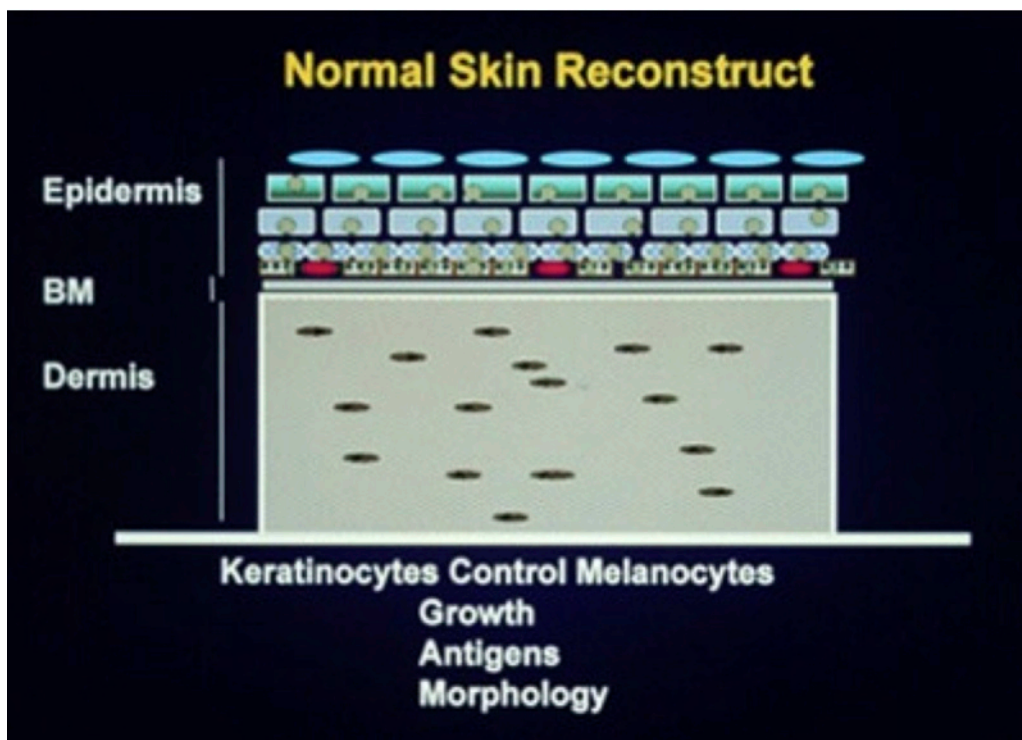


Fig.1

Melanocyte Reconstruct Media (MRM)

Components	Medium I (Days 1-2)	Medium II (Days 3-4)	Medium III (Day 5+)
Keratinocyte Serum-Free Media		---	
Bovine Pituitary Extract (BPE)		60 ug/ml	
Dialyzed Fetal Bovine Serum		2%	
bFGF (recombinant human bFGF- obtained from <i>E. coli</i>)		1.1 ng/ml	
ET-3		100 nM	
SCF 10 ng/ml		10 ng/ml	

Epidermal Growth Factor (EGF)	1 ng/ml	0.2 ng/ml	None
CaCl ₂	None	None	2.4 mM

Sterilized by using 0.22 µm filter

Notes: All concentrations and percentages are written as final concentrations in the media. All concentrations of ingredients stay constant for Medias I, II, III except for EGF and CaCl₂ which vary as indicated above.

For incorporation of melanomas, cells were seeded together with keratinocytes onto dermal reconstructs at a 1:5 ratio of melanoma cells:keratinocytes. Culture conditions were the same as for reconstructs containing keratinocytes and normal melanocytes, with modified media.

For 1L	Days 1 and 2	Days 3 and 4	Days 5+
	Epidermalization I	Epidermalization II	Cerrif. III medium
DMEM	725	725	474
F12	240	240	474
L-glutamine (200mM)	20	20	20
Hydrocortisone (269 µ/ml)	2	2	2
ITES	2	2	2
OP	2	2	2
Adenine (90mM)	2	2	2
CaCl ₂	2	2	2
Triiodothyronine	2	2	2
Progesterone	2	2	-
Serum	1 (CNBCS)	1 (NBCS)	20 (NBCS)

*All volumes are in ml

ITES=Insulin, Transferrin, Ethanolamine, Selenium

Stock concentration: 500X (Insulin: 5 mg/ml, Transferrin: 5 mg/ml, Ethanolamine: 5 mM, Selenium: 5 g/ml)

OP=O-Phosphorylethanolamine

Stock concentration: 0.05 M (7.05 mg/ml)

Final concentration: 10⁻⁴ M (0.1 mM) (14.1 g/ml)

Adenine

Hydrochloride, min. 99%

Stock concentration: 90 mM (15.5 mg/ml)

Final concentration: 0.18 mM (31 g/ml)

CaCl₂

Stock concentration: 500X

Final concentration: 2.4 mM 0.5 mM

Preparation: Dissolve 3.55 g in 20 ml of ddH₂O and filter-sterilize

Triiodothyronine

Stock concentration: 10 nM (6.5 g/l)

Final concentration: 20 pM (13 ng/l)

Progesterone

Stock concentration: 2 nM (500X) (635.9 ng/ml)

Final concentration: 4 pM (1.27 ng/ml)

NBCS=Newborn Calf Serum, Non-Chelated

Chelex 100

Preparation: Mix 10 g of Chelex with 100 ml of NBCS and stir for 3 hours at 4°C, filter-sterilize supernatant (top layer)

3. MELANOBLAST MEDIA

Components	Melanoblast	
	[C _{final}]	V = 512 ml
MCDB153	87%	87 ml
Fetal Bovine Serum (FBS)	2%	2 ml
Chelated FBS	10%	10 ml
L-glutamine (200mM)	5 µg/ml	1 ml
Cholera toxin	15 µg/ml	50 µl

bFGF	0.5 ng/ml	200 µl
ET3	100 nM; 264 ng/ml	200 µl
SCF	1.68 mM; 10 ng/ml	100 µl

Sterilized by using 0.22 µm filter

4. MELANOMA ISOLATION MEDIA

Tu2% is used for majority of melanoma cell lines.

Components	Tu2%		
	[Cfinal]	V=511 ml	V=102 ml
MCDB153	80%	400 ml	80 ml

Leibovitz's L-15	20%	100 ml	20 ml
Fetal Bovine Serum (FBS)	2%	10ml	2 ml
Insulin (Bovine) 5 mg/ml	5 µg/ml	0.5 ml	0,1 ml
CaCl ₂ 2M	1.68 mM	0.42 ml	0.084 ml

Sterilized by using 0.22 µm filter

Mel2% is used for establishing melanoma cell lines and culturing WM 3523/3523 and WM373

Components	Mel2%	
	[C _{final}]	V=512 ml
MCDB153	80%	400 ml
Leibovitz's L-15	20%	100 ml
Fetal Bovine Serum (FBS)	2%	10 ml
Insulin (Bovine) 5mg/ml	5 µg/ml	0.5 ml
Bovine Pituitary Extract (BPE) 13mg/ml	15 µg/ml	0.6 ml
Epidermal Growth Factor (EGF) 5 pg/ml	5 ng/ml	0.5 ml
CaCl ₂ 2M	1.68 mM	0.42 ml

Sterilized by using 0.22 µm filter

Mel/TPA is used for cell line WM1650

Components	Final Concentration
RPMI	-
FCS	6%
Cholera toxin	10 ⁻¹² M
TPA	10 ⁻⁷ M

Culturing WM 1650

This line is an EXTREMELY slow grower and even more painful to defrost:

- Use a 1% gelatin coat for at least 10 minutes in the incubator before defrosting the cells onto your flasks.
- Use 1% gelatin coats each time you split the cells. We recommend defrosting these cells into either a T25, or 2 wells of a 6-well plate.
- Please be patient with this line and refresh media 2x/week. We would not recommend splitting the cells higher than a 1:2 ratio. Also, please try not to split cells until they are 90-95% confluent. They sustain themselves better when they have good cell-cell interaction.

5. VESSEL RECONSTRUCTION

A 3D angiogenesis model in vitro has been developed to study migration, survival, proliferation and differentiation of human microvascular endothelial cells (HMVEC) in a fibroblast (or tumor cells) and collagen environment. The branching three-dimensional capillary-like structures are directly dependent on fibroblast-endothelial cell contact and are not achieved when fibroblasts (FF) are replaced by the different stages of melanoma cells that include radial growth, vertical growth and metastatic stages. Vascular endothelial growth factor (VEGF), when overexpressed in fibroblasts, can stimulate HMVEC proliferation. Neutralizing antibodies against VEGF and blocking antibodies for VEGF-receptor 2 could inhibit but not completely obliterated capillary network formation, suggesting that the VEGF signaling pathway is important but not exclusive. The other fibroblast-derived soluble factors and fibroblast-endothelial cell contact are essential for endothelial cell survival and differentiation.

Method

Day 1: HMVEC cells are plated on bovine collagen I coated 24-well-plate and grow to about 80% confluence.

Day 2: Add 150ul/well collagen I mixture as the first acellular layer on a HMVEC monolayer. 450ul/well collagen I mixture with 2.5-5X10⁵/ml fibroblasts are overlaid as the second cellular layer. 1ml/well EBM-2 media is added on top and media is changed every 2 days.

Day 5-7: Harvest reconstructs and stain HMVEC cells with anti-CD31 or anti-vWF VIII antibody.

Reagents

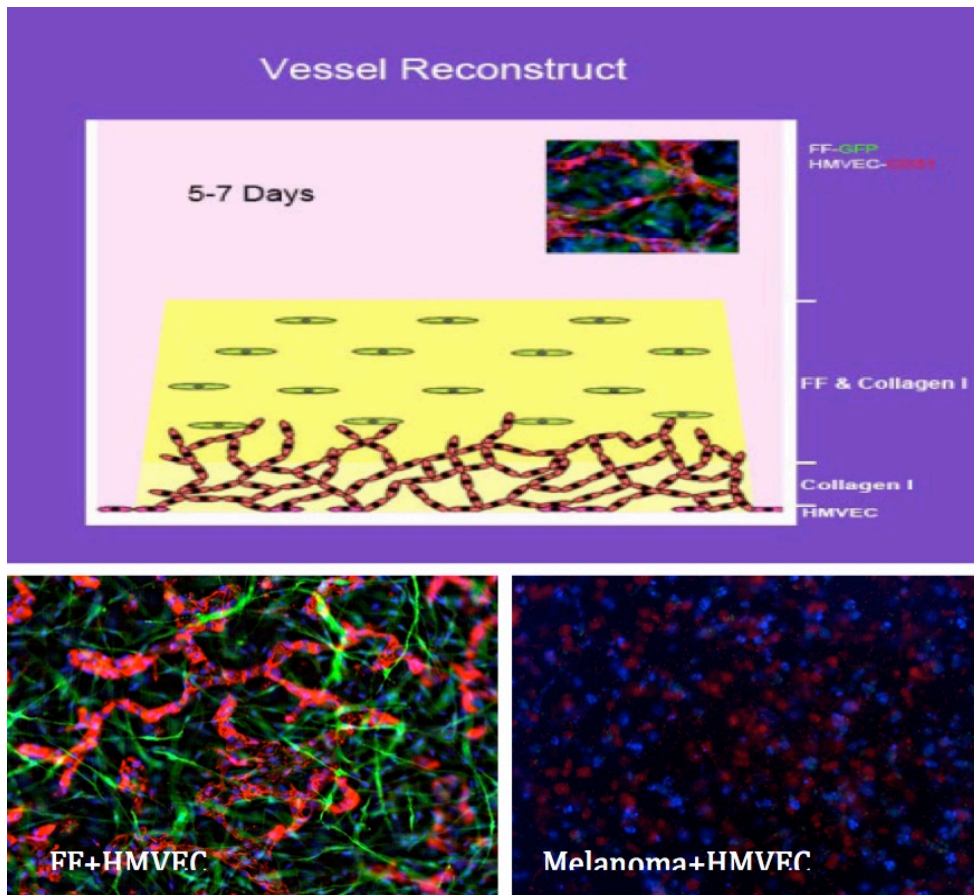
Component	Acellular layer collagen 1ml for 6 wells	Cellular layer collagen 8mL for 12 wells
Enriched 10x M199	110ul	732ul
L-glutamine	8.3ul	68ul
FBS	100ul	800ul
Sodium Bicarbonate (7.5%)	29.5ul	226ul
Collagen I (1.012mg/mL)	760ul	6220ul

Notes: 1.Enriched 10x M199 media is 10xM199 with vitamin C (50ug/ml), heparin (100U/ml)

and FBS (1%).

2. Keep all reagents on ice.

3. Change the amount of Sodium Bicarbonate according to the color of collagen mixture (like straw yellow).



6. MELANOMA STEM CELL MEDIA

1. Culture mouse embryonic fibroblasts (MEF) derived from E13-14 of CF-1 mouse.
2. Expose these cells to hES medium for 24 hours to produce condition medium (hESCM).
3. To prepare 1 liter of melanoma stem cells medium (hESCM4), mix hES and hESCM at the reation 30:70.
4. this medium can be used with or without adding fresh bFGF.

Components	HES		
	[Cfinal]	V=250 ml	V=100 ml
DMEM/F-12	80%	200 ml	80 ml
KnockOut™ SR	20%	50 ml	20 ml
L-glutamine	100mM (β-mercaptoethanol 0.1mM)	1.25 ml	1 ml
MEM Non-Essential Amino Acids Solution 10 mM	1%	2.5 ml	1 ml
bFGF (2 µg/ml)	4 ng/ml	0.5 ml	0.2 ml

Sterilized by using 0.22 µm filter

Components	HESCM4		
	[Cfinal]	V=250 ml	V=100 ml
HESCM	70%	175 ml	70 ml
HES	30%	75 ml	30 ml

Cryo-preservative Medium for Melanoma Stem Cells (2X)

KnockOut™ SR	30%
DMSO	10%
HES	10%
HESCM4	50%

To freeze Melanoma Stem Cells: 1V of 2X Cryo-preserved medium + 1V of hESCM4