10/23/2020

Drug Induced Toxicity

Drug: Cyclosporine A
↳ immunosupressant but very toxic to liver (can't be administered in ↑ doses)

Cells: Tubular epithelial cells

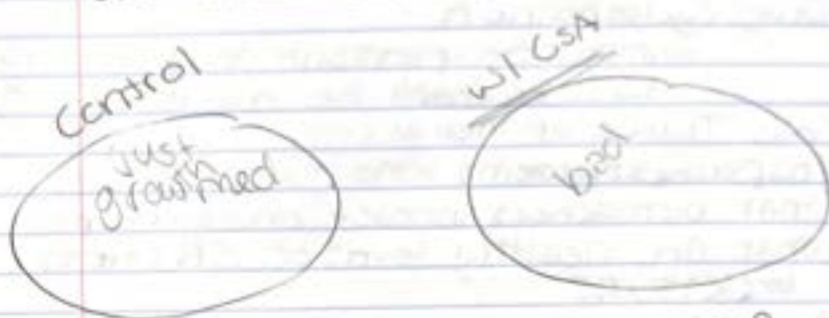
Chaperones: proteins who guide folding

→ What biomarkers increase chaperone production?

→ What are healthy levels of ER stress
↳ MEASURE

Biomarkers of ER stress: ?

do I have enough cell culture?



cell media
↳ growth medium to support growth

prove CS

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Important
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prove CSA-chap-nano w/ FTIR

how to measure biomarkers inside cell?
↓and
drug-chap-nano

which biomarkers are not genes
★dont want gene expression
↳ use Tht (florescence)
+ concentration of Ca^{2+}
ions in cytosol (should
be ↑ w/ ER stress)

Important w/ cell culture
- work on one cell line at a time
- use disinfectant (ex 70% alcohol)
- seperate bottles for each culture
- no card board packaging around cells
- test for presence of mycoplasma (contamination)

- don't let cell cultures become fully confluent
  ↳ % of vessle covered by cell
  ★should be sub-culture at 70-80%
  ↳ can use an adverted phase microscope to address density of cells

★focus on color of the media
↳ cells should have pH ~~of~~ around 7.2

Revival of cells from frozen state:

→ have medium ready BEFORE

1. warm medium in water bath
2. place cells in waterbath at 37°C
   - DONT use incubator to thaw
   - DONT get water near cap of cell container (might contaminate
3. remove cell vial from waterbath when no ice cristals are remaining
4. ? wipe vial w/ 70% alcohol solution + transfer to softey cabinet
5.

Do I need a cell suspension culture (one that reproduces uniform clones for lab use?)

Upon arival of

1. immediatly remo transfer to liqu
   ↳ ALWAYS k ice, never

☆ figure out w lines from

2. culture cells set to 37°C
3. waterbath a
4. need right
5. ☆ flask is cell-
   → do in

BE SUPER C machines, surfa

Quick Cell

☆ should be c EVERYDAY

Get how to determine cell density

should be media sho yellow w/ phase co

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solution + transfer to

Upon arival of cells:
① immediatly remove from dry ice package and transfer to liquid nitrogen
↳ ALWAYS KEEP VIALS exposed to dry ice, never any room temp
☆ figure out where to read cell guidelines from place BEFORE they come
② culture cells in humidifyed incubator set to 37°C and 5% CO_2
③ waterbath also to 37°C IMPORTANT
④ need right media for cells
☆ ⑤ flask is cell-culture treated
→ do I need to buy this?
BE SUPER CLEAN wipe down bottles, machines, surfaces, etc.

Quick Cell Check / Cell maintainence
☆ should be checked under microscope EVERYDAY (no contamination, only a few dead, + growing as expected)
should be mainly attached to bottom of dish
media should be pink-orange in color
yellow = contamination
w/ phase contrast see cells better

(next pg)

how to determine cell density

look at normal renal epithal cells to compare

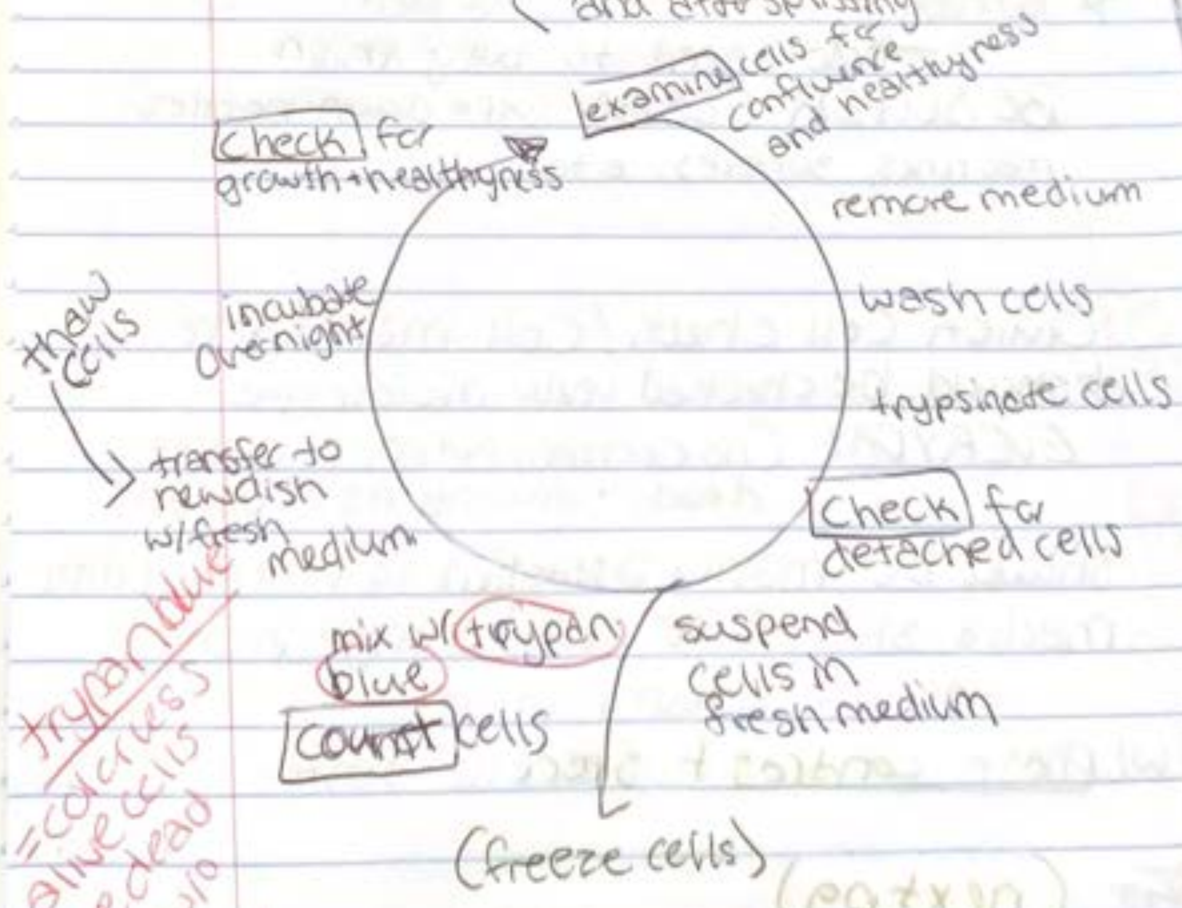
epithal cells grow adherent

① pipet medium off cells
② carefully wash cells up to 3 times w/ 5-10 mL of prewarmed PBS w/o calcium and magnesium

to seperate cells for subculture
③ use trypsin combined w/ Ethylenediaminetetraacetic acid (EDTA)

to seperate cells from eachother

④ examine the cells at beginning, during trypsination, cell counting and after splitting



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How do cells react to extra GRP78/94?
→ overexpession?
How can I get GRP78 a polar large prot to
cross the membrane?
How does CsA usually enter the cell
→ through facilitated diffusion
Will nanos work for this?

need:
- ☐ scan of ; snap (in ftir)
- ☐ scan of ; nanos (ftir) → doing that now
- ☐ laser thing of nanos
- ☐ scan of chap + nano
- ☐ laser of chap + nano

questions abo cell culture:

- understand ① arival - thawing
  ② culturing
  ③ subculturing
  ④ expirament

④ at what point of cells lifespan can you expirament / add medication?

① do you have to thaw / use / culture all cells you recieve?

②/③ how to determine population doubling time

do i use a compound microscope for the hemocytometer?

Experiment

Upon arrival

growth med: 4°C in dark

subcult. reagents: -20°C at arrival, 4°C after thawing

→ THAW overnight in fridge

*only stable for 2 weeks after thaw

Thawing of Cryopreserved Cells

1) remove from liquid nitrogen
2) turn vial cap a quarter to release liq nit then retighten cap
3) thaw quickly by placing lower 1/2 of vial in 37°C water bath
   *watch closely while thawing
4) take out of bath when only small amt of ice is left in vial
   *don't thaw cells completely
5) decontaminate vial exterior w/ 70% alcohol in Bio. Saftey Cabinet
6) remove vial cap carefully - don't touch rim of cap or vial
7) resuspend cells by gently pipetting cells 5 times w/ a 2ml pipette
   *don't pipette too vigorously for foaming
8) pipette cell suspension (1ml) from vial into T-75 flask containing 15 ml growth med
9) cap flask + rock gently to distribute cells evenly
10) place T-75 flask in 37°C, 5% CO_2, humidified incubator
   → loosen flask, don't disturbe for 24 hours

Thawing (cont.)

11) change fresh growth med after 24 hrs or overnight to remove traces of DMSO
12) change med every other day until 45% confluence
13) double volume of growth med when culture is >45% confluent OR for weekend feeding

When confluent

- measure confluency via hemocytometer
- measure viability via LDH assay

SUBCULTURING

(remove subcult. reagent kit from -20°C freezer and thaw in fridge overnight)
↳ swirl to make a homogeneous solution

★ decontaminate outside of bottles w/ 70% alc in a sterile hood

→ ① trypsinize cells

1) remove med by aspiration
2) wash w/ HBSS + remove by asp
3) add 8ml Trypsin/EDTA into T-75 flask and rock it so it covers all cells
4) re-cap tightly + monitor under inverted microscope for 60 SECONDS at room temp
   * cells should begin to look rounded and slide
5) aspirate trypsin
6) recap and place in incubator for 60sec - 1min
7) hit side of flask against palm to detach most now-rounded cells

Cells should be passaged/subcultured when they cover the plate or the cell density exceeds capacity of medium
* for Everypass: 1.2 x 10^6
for 12 well: .5 x 10^6
for T-25 flask: 2.5 x 10^6

[illegible] cells

[illegible] for 30 sec + repeat step 7

[illegible] neutralizing solution

[illegible] pipette) [illegible] into a 50ml [illegible] tube

[illegible] 300-5ml Trypsin neutralizing sol + trypsin [illegible] tube

[illegible] under microscope. if >50% [illegible] repeat #5-9

13) centrifuge conical tube at 220xg for 5 min
14) aspirate supernatant (top liquid) w/o disturbing pellet
15) flick tip of tube to loosen the cell pellet
16) resuspend cells in 5ml growth med by pipetting cells to break up clumps

→ ② count cells

- use LDH viability assay
- use hemocytometer

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-25 flask 2.8 x 10^6

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Subculturing (cont)

IF NOT DETACHED

8) place in incubator for 30 sec + repeat step 7
9) 5ml Trypsin neutralizing solution
10) transfer (w/ pipetter) cell susp into a 50ml sterile conical tube
11) rinse flask w/ add. 5ml Try neut sol + transfer to same conical tube
12) examine flask under microscope, if > 20% cells left, repeat #2-9
13) centrifuge conical tube at 220xg for 5min to pellet cells
14) aspirate supernatant (top liquid) w/o disturbing cell pellet
15) flick tip of tube to loosen the cell pellet
16) resuspend cells in 2ml growth med by pipetting the cells to break up clumps

→ ② count cells

- use LDH viability assay
- use hemocytometer

get proliferating cells

ways to study them

study for 1-4 days

① adhesive properties evaluated visually

↳ counted 30, 60, 120, 180, 1440 min of culturing

② proliferating

1) trypsinized + add Trypan Blue
2) counted w/ hemocytometer + used under a microscope

(blue cells = dead, non blue = alive)

③ Tht for ER Stress (no flow = no stress)

5 uM (micromole)/L concentration

↓ use light microscope + take

to absorb morphological changes

(Thioflavin T)

At what density are these put in t-25 flask?
540,000

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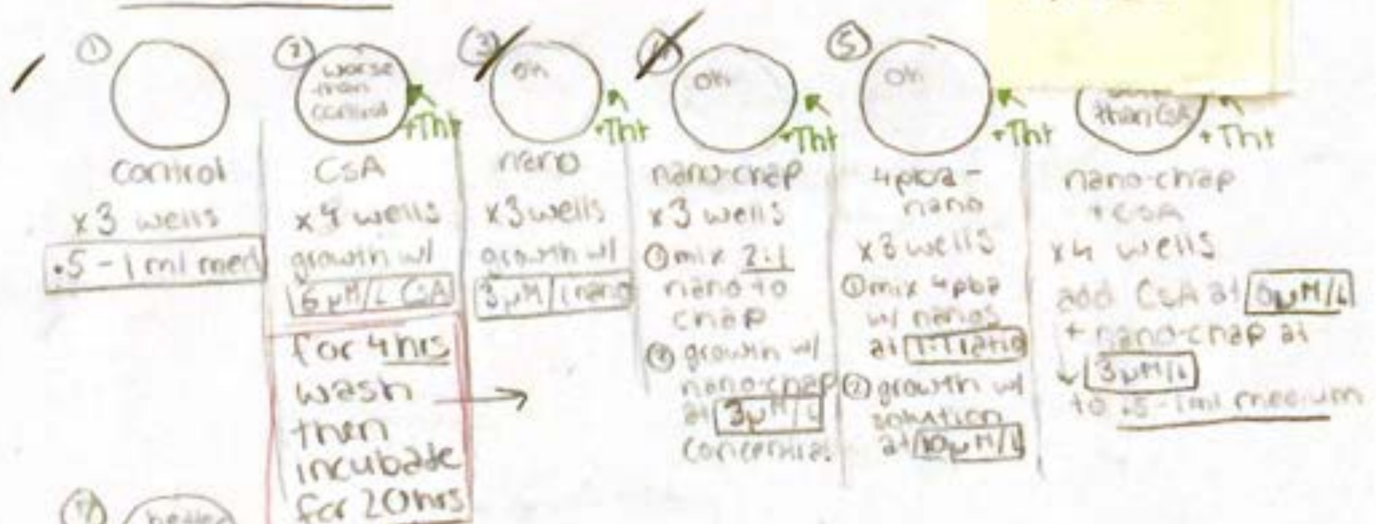
nonviab...
AND ...
↓
do 1 fir...
or doe...
w/ flow

Cell cultures - 24 well plate



⑦ better than CSA +Tht

nano-4pba + CSA

x4 wells

add 4pba + nano at 1:1 ratio at 10 µM/L

+ CSA at 6 µM/L

to .5 - 1ml med

Tht on back (for 10 mins)

1	3	4	5	6	7
1	3	4	5	6	7
1	3	4	5	6	7
2	2	2	2	6	7

after 24 hr incubation (4 w/ drug/compounds +
20 w/o → just fresh growth
AFTER x2 wash w/ HPBSS)

add Tht flourescence
5μM/L concentration & protect tht from light
for 10 mins then read

get proliferating cells
confluency of T-25 flask
↳ measure by hemocytometer + trypan blue (under microscope)

SUB-CULTURING: ① trypsinize cells
1) remove medium by aspiration
2) wash w/ HBSS + remove by asp.
3) add 3ml Trypsin/EDTA into T-25 + rock to ensure it covers all cells
4) re-cap tightly + monitor w/ inverted microscope for 60 seconds (should begin to look round + should slide)
5) Aspirate Trypsin
6) re-cap + place in 37°C incubator for 30sec - 1 min
7) hit side of flask against palm to detach most now-rounded cells
IF NOT detached: 8) place in incubator for 30 sec + repeat step 7
9) 6ml Trypsin neutralizing solution
10) transfer (w/ a pipette) cell suspension into a 50ml sterile conical tube
11) rinse flask w/ additional 5ml Try neut sol. + transfer in same conical tube
12) examine flask under microscope, if >20% cells left repeat 2-9
13) centrifuge conical tube at 220xg for 5 min to pellet cells
14) aspirate supernatant (top liquid) w/o disturbing cell pellet
15) flick tip of tube to loosen the cell pellet
16) resuspend cells in 2ml growth medium by pipetting the cells to break up clumps

② count cells
1) 15μl cell suspension into an eppendorf tube
2) 15μl trypan blue
★ dilution factor of 2 bc its a 1:1 ratio of cells to tryp

hemocytometer:

1) on 5x5 grid, count 4 corner grids
• count only unstained cells (alive)
• count only stained cells (dead)
2) take average cell count of each set of corner square
3) multiply by dilution factor
4) multiply by 10^4 to get cells per mL

calculation:
square 1# + 2# + 3# + 4# = total cells
/ 4 squares
= ave × dilution factor
then × 10^4
= _ × 10^4 cells per mL

to calculate viability:
1) add live + dead cells to = total cell count
2) divide live / total to calculate % viability

★ cells used in hem are no longer used

transfer cells to 24well plates

* into well culture dish and allow to adhere overnight before incubation w/ treatments

and once cells are in wells, use cross technique (gently back and forth) or figure 8 (in 8 pattern) to evenly distribute in well

do .5 to 1.0 ml growth med per well

Seeding density = $.05 \times 10^6$
Cells at confluency = $.24 \times 10^6$

*no need to precoat wells

Save some cells, add to T-75 flask to grow to have a reserve in case

Seed cells for 48 h[illegible] w/ 1 ml medium

after, begin experiment and us .5ml med + compounds

*bc let incubate for 48 hrs, need lower amt of cells to begin

gold nano prodedure

use 3μg/ml concentration + ~~[illegible]~~ for 3 hours

- when cells are growing exponentially
- aspirate growth medium
- add growth med w/ 3μg/ml conc of AuNPs
  → incubate for 3 hours
- after, aspirate solution
- wash twice with HBSS
- add growth medium + incubate cells for 3 hours

☆ → now study cells according to adhesive, proliferating (so count these cells) and ThT response

for chap + nano covalent bonding

2:1 ratio of gold to chap

- gently shake for 1 minute and let sit for 5 minutes before using

☆↑ follow steps above (for just nanos)

→ comes in powder form

ThT procedure

→ product sheet says methanol (.1% w/v) (should be grams/mL?)

- dissolve in .5ml 70% ethanol
- then dilute in 5.5 mL media to create a 5mM stock solution
- further dilute in media to a final 5μM concentration

Imaging of fluorescent of live cells

- taken w/ 500ms exposure under a 10x objective

↓

cells were manually counted

☆ in cells w/o ER stress, background fluorescence was detected

1) aspirate growth med
2) wash x1 with HBSS
3) apply diluted ThT-growth
   → incubate for 30 minutes at 37°C w/5% O_2

☆ protect tht from light

to dilute into growth med

① gold nanos already a liquid
→ gold + chaps also
} just dilute to proper concentration w/in growth med

② Tht is powder - in .1% methanol
③ CsA is powder - in .1% DMSO
④ 4-PBA also
} then make a working conc.

↑ otherwise higher %s are toxic

* if use DMSO, perform assays <u>in duplicate</u>
① w/ DMSO only
② w/ DMSO and compound

10/13 → 0001, 0002 - 0005 = 240 (yellow magnif)
→ 0006 - 0010 = .25 (other, thicker yellow)
→ 0011 - 0019 = .40 (only silver)

10/14 → 1401 - 1407 = silver only
→ 1408 - 1412 = yellow thin
→ 1413 - 1414 = red thick / small
→ 1415 - 1420 = yellow thick

10/14 subculturing:
→ 1424 - 1426 = thick yellow
→ 1423 = silver
→ 1421-22 + 1427 = thin yellow

10/15 → 1528 - 1537 — silver thin
→ 1538 - 1541 → thin yellow
→ 1542 - 48 = red thick
→ 1549 - 1553 = yellow thick

10/18 → 1854 - 1859 → thick yellow
+ 1862
→ 1863 - 1869 → silver thin

10/19 → 1901 - 1908 → thin yellow
1909 → red
1910 - 1923 → thick yellow
1924 - 1953 → silver

10/20 → 2018-2026 (silver)
→ 2027-2044 (silver w/ flar filter) > W4A
→ 2045-55 (silver)
→ 2056-2060 (silv w/ flar) > W~~4~~3A
→ 2061 - 2064 (silv)
→ 2065 - 2066 (silv w/ flar) > W2A

W1 = barely any cells left

10/21 → 2167-2181 (silver)
2182-2191 (yellow thin)
2192-2104 (silver flar) > W4
21105-21107 > W1

10/22 → 22108-22118 (silver + flar) W4
→ 22119-22129 (silv flar) W3 (W2 = not good no cells basically)
→ 22130-22135 (silv flar) W1
→ 22136-22157 (silv) W4
→ 22159-22161 (silv) W1
~~→ 22162-22164 (silv)~~ to FLASK