A RadoNorm Short course entitled:
CELET: Cellular effects of high and low LET ionising radiation – introduction to radiation biology

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This is the complete protocol that we use at the SU for culturing and fixing human peripheral blood lymphocytes for the analysis of aberrations and micronuclei. It is presented here for the sake of completeness. During the "aberrations exercise" you will learn how cells are harvested for analysis of aberrations. To this end we will use cells from the human cell line RPE-1 (hTERT-immortalized retinal pigment epithelial cells). These cells are constantly used in our lab and the harvesting procedure is similar as for lymphocytes. The advantage of using a cell line is that we do not need to collect blood from a donor before the exercise, which would require a separate ethical permit. RPE-1 cells grow attached to a dish. They will be detached by trypsin before the exercise.

For this exercise we will compare two methods of hypotonic treatment: 0.075 M KCL and 1% sodium citrate. Groups 1 and 2 will use KCL and group 3 and 4 - sodium citrate. The aim of the exercise is to compare the quality of the slides following the two hypotonic treatments. KCL is more aggressive than NaCitrate, leading to the dispersal of chromosomes on slides. The quality of slides will be compared by scoring the mitotic index of cells treated by either hypotonic.

The protocol

Harvesting cells for the analysis of aberrations

- Spin cultures 10 min, 1000 r/min
- Aspirate supernatant, leave about 0.5 ml pellet
- Slowly add (mix by gentle vortexing) 5-7 ml of warm (37^{o}C) hypotonic solution:
  0.075 M KCL (groups 1 and 2)
  1% sodium citrate (groups 3 and 4)
- Place for 10 min in a water bath at 37^{o}C
- Spin 10 min, 1000 r/min
- Aspirate supernatant, leave about 0.5 ml pellet
- Slowly add (mix by vortexing) 5-7 ml of ice cold (0^{o}C) fixative
- Spin 10 min, 1000 /min
- Perform 2-3 washes with fixative – supernatant must be clear
- Aspirate supernatant leaving pellet in 0.1 - 0.5 ml of fixative
- Mix the pellet and drop ca 50 µl in separated drops on degreased slide
- Dry slides for ca 10 min, stain with 10% Giemsa

In E214, analyse the mitotic index (% of mitotic cells) on the slide by counting ca 500 nuclei, Report the results to group 1.

Culture of human peripheral blood lymphocytes

For analysis of aberrations and micronuclei

Medium

296 ml RPMI 1640 (80%)

74 ml foetal calf serum (20%)

5.5 ml PHA (GIBCO 10576 - 015)

1.85 ml antibiotics (GIBCO 15245 - 012)

Sum: 377.3 ml

Freeze medium (-20° C) in culture tubes, In portions of 4.5 ml (Greiner 19 11 60).

Blood collection and culture

Collect blood into Sarstedt syringe or Vacutainer.

Keep at room temperature until culture onset

Prewarm medium to 37°C

Add 0.5 ml blood to 4.5 ml of warm medium, mix gently

Add BrdU (80 µl STOCK/ 5 ml medium)

Keep at 37° C, 5% CO_2. If no CO_2 is available, close tubes tightly.

Chromosomal aberrations

Add Colcemid after 48 - 50 hours of culture time - 25 μl / 5 ml medium (end concentration = 0.05 μg/ml)
Harvest cells after 2 - 4 hours
Stain by FPG (pg 5) if needed and if BrdU was used (pg 6) or simply with Giemsa.
FPG staining is used when cells in the first post-treatment mitosis must be identified.
An alternative protocol is to add Colcemid at reduced concentration already after 24 h of culture time.

PCC: add 25 μl Calyculin A solution / 5 ml medium 30-60 min before harvest.

Harvesting cells for the analysis of aberrations

- Spin cultures 10 min, 1000 r/min
- Aspirate supernatant, leave about 0.5 ml pellet
- Slowly add (mix by vortexing) 5-7 ml of warm (37°C) hypotonic solution - 0.075 M KCL
- Place in a water bath or incubator (37°C) for 15 min
- Spin 15 min, 1000 r/min
- Aspirate supernatant, leave abort 0.5 ml pellet
- Slowly add (mix by vortexing) 5-7 ml of ice cold (0°C) fixative
- Leave overnight in refrigerator or proceed with next step
- Spin 10 min, 1000 /min
- Perform 2-3 washes with fixative – supernatant must be clear
- Aspirate supernatant leaving pellet in 0.1 - 0.5 ml of fixative
- Apply 2-3 drops of pellet on degreased, cold (fridge) slides. Check for cytoplasm contamination. If contaminated, add few drops of acetif acid to pellet, mix, try again. Try applying n wet slides. Make 2-4 slides per pellet.
- Dry slides overnight. For FPG staining age for 2-4 days. No aging necessary for pure Giemsa staining.

Micronuclei

Add Cytochalasin B (Cyt B) after 44 hours of culture time - 85 μl / 5 ml medium
Harvest after 72 hours of culture time

Harvesting cells for micronuclei

- Spin cultures 15 min, 900 r/min
- Aspirate supernatant, leave about 0.5 ml pellet
- Slowly add (mix by vortexing) 5-7 ml of warm (37°C) hypotonic solution 0,14 M KCL
- Leave at room temperature for 5 min
- Spin 15 min, 900 r/min
- Aspirate supernatant, leave abort 0.5 ml pellet
- Slowly add (mix by vortexing) 5-7 ml of fixative I
- Leave at room temperature for 5 min
- Spin 10 min, 1000 r/min
- Aspirate supernatant, leave abort 0.5 ml pellet
- Slowly add (mix by vortexing) 5-7 ml of fixative II
- Leave at room temperature for 5 min
- Spin 10 min, 1000 r/min
- Perform 2-3 washes with fixative – supernatant must be clear
- Apply 2-3 drops of pellet on degreased, dry slides (room temperature).

Dry slides (best results when dried overnight). Stain with Giemsa.

Isolation of lymphocytes

1. Place 10 ml of Histopaque (Sigma; no. 1077-1) at the bottom of a 50 ml tube
2. Place 9 ml of blood on top of Histopaque
3. Centrifuge at 800 g for 15 min, NO BRAKE!
4. Aspirate the visible ring and collect in a new 50 ml tube. Be careful not to collect any Histopaque from below the ring!
5. Add 30 ml of Hanks to the collected lymphocytes and centrifuge for 15 min, 1500 rpm
6. Discard supernatant and wash again with 30 ml Hanks
7. Discard supernatant and resuspend cell pellet in 1 ml medium
8. Determine the cell density

FPG staining

(Fluorescence Plus Giemsa)

- In a copplin jar add 0.5 ml bisbenzamide (poison) to 50 ml A. dest., keep in dark.
- Stain slides for 20 min (dark)
- Wash with A. dest.
- Apply 0.5 ml PBS on slide, cover with another sidle, place on 60^0C hot plate
- Expose to UV radiation (at 60^oC) for 20 - 30 min
- Wash with A. dest.
- Stain with Giemsa (5 - 10%) for 5-10 min
- Wash with A. dest.
- Air dry

Chemicals – chromosomal aberrations

0.075 M KCL

2.8 g KCL / 500 ml A. dest.

Store at 4^0C

Fixative:

Metanol	3
acetic acid	1

BrdU (5'-Bromo-2'-deoksyuridine), Sigma B9285

STOCK solution

Dissolve 0.0031g BrdU in 10 ml PBS/RPMI 1640

Sterilise by filtration (0.22 μm filter)

Store in refrigerator (4^0C) in aluminium foil.

Bisbenzamide (Hoechst 33258), Sigma B28283 – POISON!

STOCK:

Dissolve 0.05g in 100 ml A. dest.

Store in aluminium foil at 4^0C

Working solution: mix 0.5 ml with 50 ml A. dest.

Calyculin A (Sigma C5552) – 10 µg

Dissolve content in 1 ml ethanol (water free) or DMSO

Aliguot a 25 µl in small Eppendorf tubes, store at -20^0C

Add 25 µl per 5 ml medium. Final concentration: 50 nM.

Okadaic acid

Chemicals – micronuclei

Cytochalasin B (Sigma C 6762)

10 mg dilute in 3 ml DMSO

aliquot a 50 μl in small, sterile Eppendorf tubes, store at -20^{0}C

before use dilute 50 μl / 450 μl RPMI

add 85 μl / 5 ml culture medium. End concentration = 5.6 μg / ml

0.14 M KCL

5.2 g KCL / 500 ml distilled water.

Keep at 4^{0}C

Fixative I:

methanol	12
0.9% NaCl	13
acetic acid	3

Fixative II:

methanol	4
acetic acid	1

How to score micronuclei, how to calculate the dispersion index and the replication index

Micronuclei (Mn)

You score:

1. The frequency of Mn in 1000 binucleated cells. Document the number of binucleated cells that contain:

0 Mn

1 Mn

2 Mn

3 Mn

4+ Mn (4 and more)

The distribution of Mn is analysed by calculating the dispersion index (DI):

DI = VAR/M

where: VAR = variance

M = mean

2. Number of cells with different numbers of nuclei: mono, bi, tri and tetranucleated (and more) cells:

Calculate the replication index (RI) as follows:

RI = (MONO + 2 x BI + 3 x TRI + 4 x TETRA+) / TOTAL

where: MONO = number of mononucleated cells

BI = number of binucleated cells

TRI = number of trinucleated cells

TETRA+ = number of tetranucleated (and more) cells

TOTAL = number of all scored cells

Calculate the RI based on scoring 500 cells