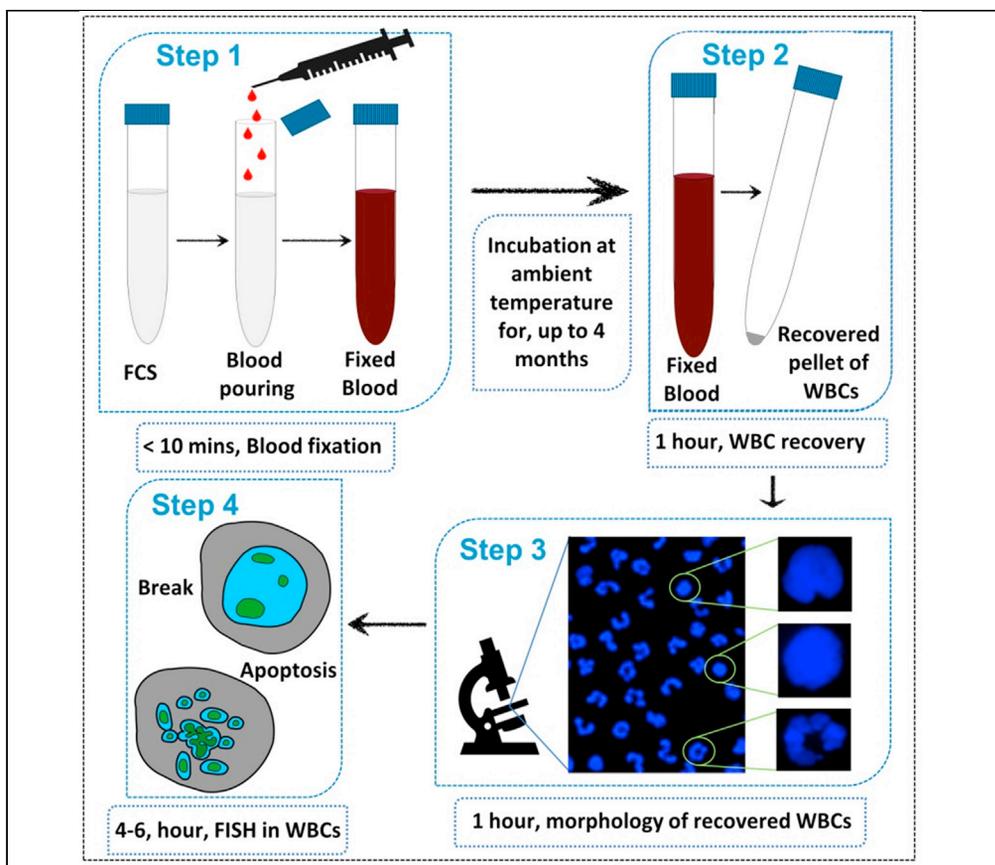


## Protocol

Protocol for one-step selective lysis of red blood cells and platelets with long-term preservation of white blood cells (human) at ambient temperature



Current protocols for storage of white blood cells (WBCs) rely on constant refrigeration. The protocol described below explains the preparation of a fixative combination saline (FCS) formulation, which allows fixation of human WBCs and lysis of red blood cells (RBCs) and platelets (at ambient temperature, 4°C–35°C) in whole blood samples in one step. FCS can be used for storing and transporting blood at ambient temperatures for up to 4 months, without altering the nuclear morphology and genomic integrity of WBCs.

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### Highlights

FCS for single-step preparation of human WBCs

FCS fixes and preserves WBCs; FCS selectively lyses RBCs and platelets

Fixed WBCs can be stored and transported at ambient temperature up to 4 months

FCS can overcome the constraint of refrigeration of blood samples

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## Protocol

## Protocol for one-step selective lysis of red blood cells and platelets with long-term preservation of white blood cells (human) at ambient temperature

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## SUMMARY

Current protocols for storage of white blood cells (WBCs) rely on constant refrigeration. The protocol described below explains the preparation of a fixative combination saline (FCS) formulation, which allows fixation of human WBCs and lysis of red blood cells and platelets (at ambient temperature, 4–35°C) in whole blood samples in one step. FCS can be used for storing and transporting blood at ambient temperatures for up to 4 months, without altering the nuclear morphology and genomic integrity of WBCs.

## BEFORE YOU BEGIN

1. Approval of institutional ethical committee must be obtained, and experiments must be executed as per the approved ethical guidelines.
2. The FCS-containing vials must be prepared, before the experiments are planned to be executed, by following the method detailed below. If Phosphate Buffered Saline (PBS) is being prepared in the laboratory from its constituents, it must be autoclaved. In this case the preparation time may slightly increase (1–2 h).
3. A phlebotomist with a syringe or a lancet should be ready to withdraw the blood sample either by puncturing the cubital vein or by pricking the finger.
4. Volunteers must be ready to donate the blood; consent must be obtained as per ethical guidelines.

△ **CRITICAL:** Since this protocol deals with handling of human blood samples, it is mandatory to wear gloves and a face mask to avoid the possibility of any kind of bacterial/viral/fungal infections.

## KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Chemicals, peptides, and recombinant proteins		
Acetone (≥ 99.5% v/v)	Sigma-Aldrich	Cat# 179124
Glacial acetic acid (≥ 99%)	Sigma-Aldrich	Cat# A6283
Methanol (≥ 99.5% v/v)	Sigma-Aldrich	Cat# V800258
NaCl (≥ 99.5% (AT))	Sigma-Aldrich	Cat# S7653

(Continued on next page)



<b>Continued</b>		
REAGENT or RESOURCE	SOURCE	IDENTIFIER
KCl (≥ 99.0%)	Sigma-Aldrich	Cat# P9541
Na <sub>2</sub> HPO <sub>4</sub> (≥ 99.0%)	Sigma-Aldrich	Cat# S9763
KH <sub>2</sub> PO <sub>4</sub> (99.995%)	Sigma-Aldrich	Cat# 1.05108
Sodium citrate	Sigma-Aldrich	Cat# 6132-04-3
Triton X-100 (for molecular biology)	Merck	Cat# T8787
Tween-20 (for molecular biology)	Merck	Cat# P9416
DAPI with antifade (ProLong™ Gold Antifade Mountant with DAPI)	Invitrogen	Cat# P36962
<b>Biological samples</b>		
Human, peripheral blood sample (from 5 volunteers; 3 males [age; 26, 34, and 39 years] and 2 females [age; 23 and 31 year])	Local healthy volunteers	NA
<b>Critical commercial assays</b>		
RBC lysis buffer	BioLegend	Cat# 420302
FISH probes (for chromosome pair one)	MetaSystems	Cat# D-0301-050-OR
FISH probes (for centromere of chromosome pair one)	MetaSystems	Cat# D-0801-050-OR
<b>Software and algorithms</b>		
OriginLab 2018 (9.5)	OriginLab	<a href="https://www.originlab.com">https://www.originlab.com</a>
<b>Other</b>		
Falcon™ 15 mL Conical Centrifuge Tubes	Fisher Scientific	Cat# 14-959-53A
Deionized water	Thomas Scientific	Cat# C843U58
Doble frosted glass slides	Borosil Microscope Slide	Cat# 9100D01
Cover glass	Borosil Microscope Cover glass	Cat# 9115S02
Benchtop centrifuge	Thermo Scientific™	Sorvall ST8R
Blood irradiator	BRIT, India	Blood Irradiator-2000
CO <sub>2</sub> incubator	Thermo Scientific™	Cat# 310TS
Swinging Bucket Clinical Rotor	Thermo Scientific™	TX-100
Reagent Square Glass Bottles, 500 mL	Borosil®	GL80 PP CS/10
Reagent Square Glass Bottles, 500 mL	Borosil®	Borosil 1501021
Membrane filter, 0.22 μm pore size	Millipore	Cat# GSWP04700
Micropipette 20–200 μL	Gilson, PIPETMAN LP200L	FA10005M
Micropipette, 100–1000 μL	Gilson, PIPETMAN L P1000L	FA10006M
Fluorescence microscope (with blue and red filters)	Leica	TCS SP8 confocal
Type F, immersion oil for fluorescence	Leica	Cat# 11513859
Digital water bath with fine temperature control	Equitron	Cat#7505
Thermometer	Star 49	Cat# B 34
ThermoBrite System	Abbott	Cat# 07J91-010
Freezer –20°C	Samsung	Cat# RT77KBTS
pH meter	Hanna	edge®
Forceps	Tarsons	Cat# 486000
Timer watch	Fisherbrand	Cat# 14-648-3
Glass Coplin Jar with glass lid	Borosil	Cat# SJ322
Rubber cement	Fevibond	NA
Humidified chamber (made in the laboratory)	NA	NA

**Note:** All chemicals and solvents used should be of molecular biology grade.

## MATERIALS AND EQUIPMENT

### Mixture 1: Stock FCS (0.5 mL/tube)

Reagents	Acetic acid	Methanol	Acetone
Final concentrations	64.5% v/v	32.3% v/v	2.58% v/v
Volume for 10 mL FCS	6.45 mL	3.23 mL	0.258 mL

- Storage temperature range:  $-20^{\circ}\text{C}$  to  $35^{\circ}\text{C}$ , Duration: up to 4 months)
- Storage: In dark and airtight container

**Note:** Freshly prepared Mixture 1 and 3 are clear and transparent solutions with pungent smell. It is important to store these Mixtures in a dark and airtight container to avoid photo chemical reactions and evaporation of its constituents.

**Note:** Glacial acetic acid and acetone are toxic to the skin, therefore it is recommended to use hand gloves during preparation and handling of FCS. Glacial acetic acid and acetone are volatile in nature, therefore it is also recommended to prepare FCS in fume-hood and wear a face mask during preparations.

### Mixture 2: 0.8× Phosphate buffered saline (PBS) (7.0 mL/tube)

Reagent	NaCl	KCl	$\text{Na}_2\text{HPO}_4$	$\text{KH}_2\text{PO}_4$
Final concentration (mmol/L)	109.6	2.16	8.0	1.44
Final concentration (g/L)	0.64	0.16	1.136	0.192

(100 mL 0.8× PBS = 80 mL 1× PBS + 20 mL Distilled Water)

- Storage temperature:  $4^{\circ}\text{C}$ , Duration: Up to 1 Year, if sealed properly but temperature before use should be in the range of  $20^{\circ}\text{C}$ – $25^{\circ}\text{C}$
- Storage: In dark and airtight glass/polypropylene/polystyrene bottle
- PBS (pH 7.4); autoclaved
- 0.8× PBS should be made just before use.

**Note:** After preparation, 0.8× PBS needs to be filtered with membrane filter, 0.22  $\mu\text{m}$  pore size, though glacial acetic acid, methanol and acetone need not be filtered (recommended to use molecular biology grade).

### Mixture 3: Working FCS (7.5 mL/tube = 0.5 mL Stock FCS + 7 mL 0.8× PBS)

Reagent	Acetic acid	Methanol	Acetone	0.8× PBS
Final concentration	4.3%v/v	2.15%v/v	0.18%v/v	93.33%v/v

- Storage temperature range:  $4^{\circ}\text{C}$ – $35^{\circ}\text{C}$ , Duration: up to 4 months
- Storage: In dark and airtight container

Composition of recovery cocktail: Same as Mixture 1

**Note:** Fixation, storage (of fixed blood sample in FCS) and recovery were carried out in conical centrifuge tubes (15 mL) made up of polypropylene material.

**Alternatives:** Glass tubes can also be used instead of polypropylene tubes.

**△ CRITICAL:** Store stock FCS solution in an airtight glass bottle; working FCS can be stored in polypropylene tubes too.

**Note:** We observed little change in the pH of blank FCS in the storage duration of 4 months at refrigerated or ambient temperature ( $4^{\circ}\text{C}$ – $35^{\circ}\text{C}$ ) and no difference was observed in its fixation attributes. Significant change in pH can be considered as an indicator of expiration of blank FCS. Long and/or leaky storage may lead to increase in the pH of FCS, owing to evaporation of glacial acetic acid, resulting in a change in the actual composition or proportion of the constituents. The acceptable bounds of pH for this protocol are 2.3–3.1. If pH goes below the

lower threshold (i.e., 2.3) it starts destabilizing the nuclear membrane of WBCs and if pH goes above the upper threshold (i.e., 3.1) it starts accumulation of degraded membrane debris of RBCs and platelets, which is challenging to remove from the pellet, thus resulting in recovery of WBCs with impurities.

**Note:** If all the constituents are added/mixed properly, in the proportions as described above, pH of the working FCS will be in the range of 2.3–3.1 (even after incubation up to 4 months). If pH is falling below and/or above the lower and/or upper threshold levels, it is recommended to discard this working FCS solution and prepare a fresh FCS working solution from the stock. It is not recommended to adjust the pH of working FCS solution by adding any acid or alkali.

**Note:** Freshly prepared FCS vials (air-tight) can be stored at ambient temperature in dark conditions, for up to 4 months before use.

## STEP-BY-STEP METHOD DETAILS

### Blood sample collection

⌚ **Timing:** 10–15 min

Once working FCS vials are ready for use, blood samples (30–500 $\mu$ L) may be collected from subjects by finger pricking, preferably using a lancet.

**Optional:** A capillary with anti-coagulant (such as EDTA or heparin) can be used for collection of blood.

**⚠ CRITICAL:** If there is a delay of more than 5 mins between collection of blood and its fixation in FCS, treat the blood with anticoagulant (heparin/EDTA).

**Note:** Blood treated with anticoagulant (heparin/EDTA) and stored at 4°C, can be used for fixation in FCS, up to 24 h after withdrawal. Frozen blood samples and/or blood samples stored below 4°C, were not tested for the fixation with FCS.

**Note:** The protocol below requires 0.5ml blood samples for each fixation.

### Blood fixation with FCS

⌚ **Timing:** 8–10 min

1. Infuse collected blood samples (0.5 mL) with 7.5 mL of FCS solution (Mixture 3), drop by drop (8–10 drops/min, with intermittent manual shaking after each drop, gentle vortexing can also be carried out, it is recommended to avoid high speed vortexing).

**⚠ CRITICAL:** Blood sample should be added slowly, drop by drop with gentle mixing, otherwise there would be a possibility of formation of small blood clots, which are difficult to dissolve.

**Note:** We previously optimized the ratio of FCS and blood volumes as shown in [Table 1](#). We compared the cell recovery for each ratio with recovery obtained by RBC lysis method, which served as a control.

The optimum dilution range of FCS: Blood, v/v is 1:0.1 to 1:0.45. Below we describe the protocol for a proportion of 7.5 mL (FCS): 0.5 mL (blood volume) (ratio of 1:0.15).

**Table 1. Optimization of Ratio of FCS and blood for fixation**

FCS : Blood, (v/v)	Remarks
1:0.1	Fixed well, solution remained normal; cell recovery was 45%–64%. Nuclear morphology and integrity of WBCs, viz., neutrophils, lymphocytes and mix cells (monocytes, eosinophils and basophils), were intact/preserved well)
1:0.15	Fixed well, solution remained normal; cell recovery was 44%–62%. Nuclear morphology and integrity of WBCs, viz., neutrophils, lymphocytes and mix cells (monocytes, eosinophils and basophils), were intact/preserved well)
1:0.2	Fixed well, solution remained normal; cell recovery was 46%–61%. Nuclear morphology and integrity of WBCs, viz., neutrophils, lymphocytes and mix cells (monocytes, eosinophils and basophils), were intact/preserved well)
1:0.25	Fixed well, solution remained normal; cell recovery was 44%–67%. Nuclear morphology and integrity of WBCs, viz., neutrophils, lymphocytes and mix cells (monocytes, eosinophils and basophils), were intact/preserved well)
1:0.30	Fixed well, solution remained normal; cell recovery was 42%–68%. Nuclear morphology and integrity of WBCs, viz., neutrophils, lymphocytes and mix cells (monocytes, eosinophils and basophils), were intact/preserved well)
1:0.35	Fixed well, solution remained normal; cell recovery was 47%–66%. Nuclear morphology and integrity of WBCs, viz., neutrophils, lymphocytes and mix cells (monocytes, eosinophils and basophils), were intact/preserved well)
1:0.40	Fixed well, solution remained normal; cell recovery was 45%–65%. Nuclear morphology and integrity of WBCs, viz., neutrophils, lymphocytes and mix cells (monocytes, eosinophils and basophils), were intact/preserved well)
1:0.45	Fixed well, solution remained normal; cell recovery was 40%–61%. Nuclear morphology and integrity of WBCs, viz., neutrophils, lymphocytes and mix cells (monocytes, eosinophils and basophils), were intact/preserved well)
1:0.5	Fixed well, solution became viscous and cell recovery was poor (23%–49%). Pellet contained dense cell debris of RBCs and platelets that was difficult to remove)
1:1	Mixture solidified (cells could not be recovered)
1:1.5	Mixture solidified (cells could not be recovered)
1:2	Mixture solidified (cells could not be recovered)

**Note:** Since the ratio of FCS to blood is important, the user may scale up or down the volumes of both (depending on their needs). Blood is to be mixed with FCS such that the final ratio remains between 1:0.1 and 1: 0.45.

- Gently shake the vials, to allow homogeneous distribution of blood cells. The RBCs and platelets will get lysed, and the WBCs will get fixed in the whole blood. These fixed blood samples can be stored and/or transported at ambient temperature for up to 4 months.

**Note:** FCS has been tested for its stability up to 4 months in ambient conditions (in dark and temperature range of 4°C–35°C). The fixed sample may be stored in FCS such that the total storage time of FCS does not exceed the upper limit of 4 months (i.e., if 2 months old FCS is being used for the fixation of the blood, it can be stored for 2 more months).

- Properly fixed blood sample will be a dark maroon homogeneous mixture. It contains fixed WBCs and lysed RBCs and platelets. Fixed sample needs to be stored in the dark and in an airtight vessel/tube.

pH of FCS fixed blood (500  $\mu$ L blood in 7.5 mL of FCS) sample:

- Freshly fixed blood sample (same day); pH = 3.18
- One month old fixed blood sample; pH = 3.18
- Four months old fixed blood sample; pH = 3.16

**Note:** This slight change in the pH of the fixed blood sample, does not affect the recovery of the leukocytes. After fixation, the cap should be closed airtight as leaky storage may lead to increase in the pH of fixed blood sample, owing to evaporation of glacial acetic acid. This may alter the fixation properties of the FCS.

### Leukocyte recovery

⌚ Timing: 50–60 min

- Centrifuge fixed blood samples at 1000 RPM (188×g) for 10 min (25°C, brake setting should be low, 1–2), to allow for settling of leukocytes along with the debris.
- Discard the supernatant by aspiration with a micropipette or with the help of motor/pump operated aspirator.

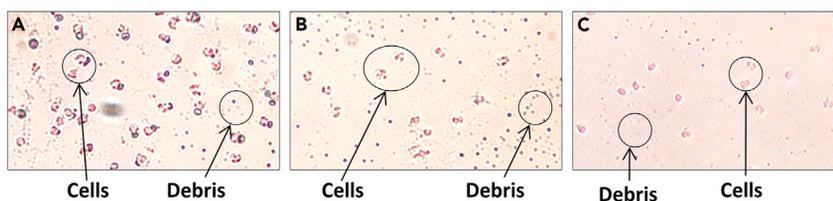
⚠ **CRITICAL:** Discarding supernatant by inverting the tube, should be avoided. The discarded supernatant must not be discharged down the drain, it must follow proper disposal procedure (read, how to dispose mixture of organic solvents).

- Resuspend the resulting pellet gently in 8 mL stock FCS Mixture 1/recovery cocktail. Recovery cocktail dissolves the debris of RBCs and platelets present in the mixture.
- Repeat washing with the recovery cocktail 2–3 times to remove all the debris and preserve the pellet. No, additional incubation is required in between, to allow lysis to occur.

**Note:** To determine the number of washes required, we recommend that you resuspend the pellet in 50 µl of recovery cocktail after the 2<sup>nd</sup> wash and take 5 µl of cell suspension and drop it on top of a glass slide, let it air dry and observe at 10 and or 40× magnification with a bright field microscope. Population of WBCs and cell debris can be easily distinguished (Figure 1). If a substantial amount of debris is observed, repeat the washes 1 or 2 more times, and recheck with the microscope. Generally, 3 washes are enough to remove cell debris, but we did not observe changes in the nuclear morphology and integrity of WBCs even after 5 washes.

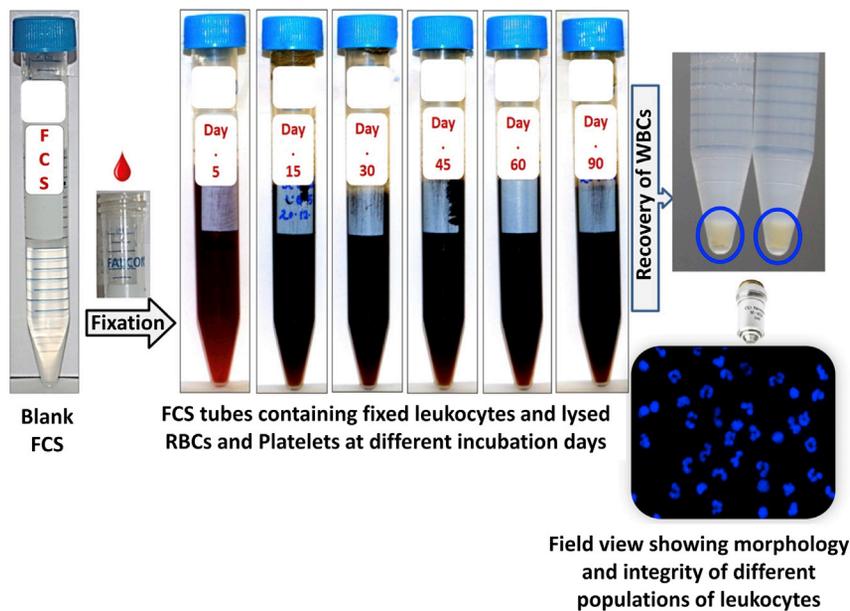
- This pellet contains all types of WBCs, which can be used as per the need of the assay.
- These recovered WBCs can further be re-suspended in stock FCS (Mixture 1) and stored at room temperature or at cold condition (4 or –20°C) for long durations. The work flow is pictorially presented in Figure 2.

⏸ **Pause point:** WBCs can be recovered anytime (within 4 months) from the FCS (Mixture 3) fixed whole blood sample. After recovery (at any time, up to 4 month), these WBCs, can be re-suspended and stored in Mixture 1, at room temperature for 5–6 days, in an airtight tube and same can be stored for, maximum up to 4 months at 4 or –20°C, without compromising its morphology and nuclear integrity (stability at 4°C and –20°C was found to be same). If WBCs are recovered after 1, 2 or 3 months of fixation, it can be stored/preserved, in Mixture 1 for 3, 2 and 1 months respectively at 4 or –20°C. WBCs recovered after 4 months of fixation, should be used within 1–2 days. In short, total storage time (either in Mixture 1 or in Mixture 3), should not exceed 4 months.



**Figure 1. WBCs with cell debris at different recovery steps**

Field view of recovered WBCs along with cell debris after (A) 1<sup>st</sup> wash (B) 2<sup>nd</sup> wash and (C) 3<sup>rd</sup> wash, with recovery cocktail (Mixture 1). It can be observed that cell debris reduced significantly after each wash.



**Figure 2.** Schematic illustration of the process of collection of blood sample in tubes containing FCS, fixation, storage, and recovery of leukocytes at ambient temperature

**Note:** For comparison and to serve as a control sample, leukocytes can also be recovered from unfixed fresh blood sample by RBC lysis method using commercially available RBC lysis buffer from BioLegend, USA, following the recommended protocol (de Resende et al., 2010).

## EXPECTED OUTCOMES

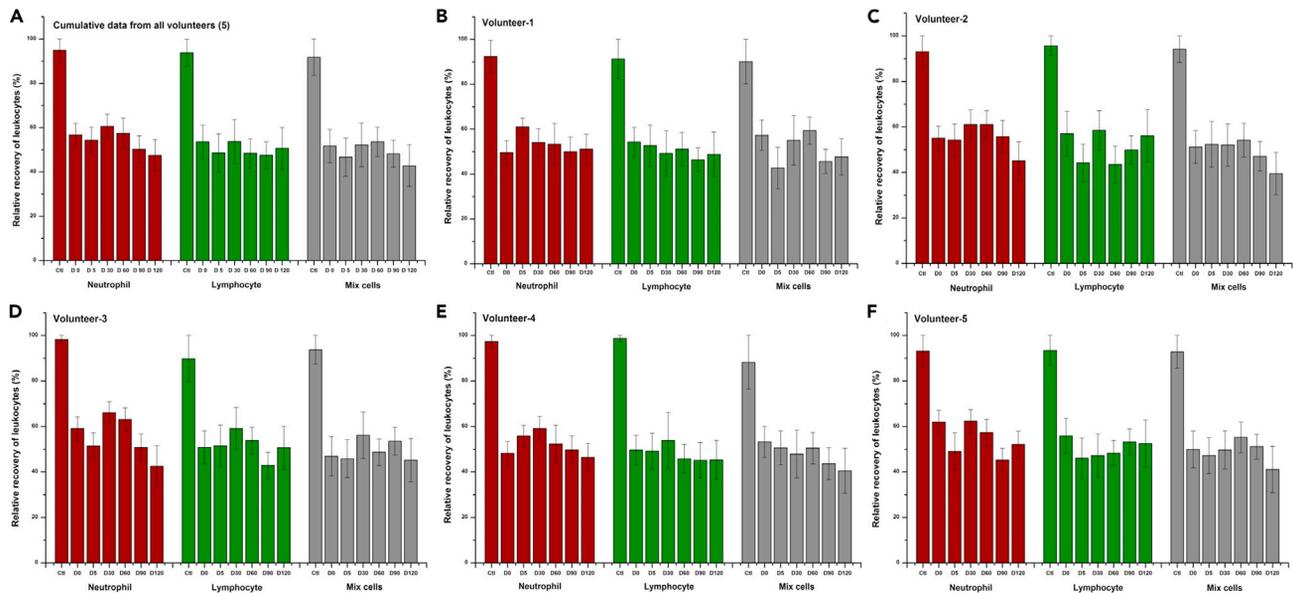
30 mL blood sample was obtained from 5 volunteers. 60 fixed blood vials (per volunteer) were prepared by mixing 0.5 mL blood, in 7.5 mL Mixture 3. In total 300 fixed vials (from 5 volunteers) were prepared and stored at ambient temperature for further investigations.

### Leukocyte recovery

Leukocytes were recovered from the fixed sample vials, at days 0, 5, and further at an interval of 10 days up to a period of 120 days (in triplicates, per individual, each time point). Differential leukocyte counts were made, manually with a hemocytometer, based on nuclear morphology (stained with DAPI) at 63× magnification, of an inverted fluorescence microscope (Leica, SP8 inverted confocal microscope, Germany), under blue filter.

More than 6000 cells were analyzed microscopically per individual per incubation time. The results were compared with the control sample.

Figure 3A, Shows the relative recovery (with respect to control sample, wherein WBCs were obtained from whole blood, by RBC lysis method) of leukocytes, from the fixed blood sample after an incubation period of 0, 5, 30, 60, 90 and 120 days (recovery of leukocytes on other incubation periods, is not illustrated in Figure 3), the error bars represent Standard Error of Mean (SEM). Figures 3B–3F, shows the relative recovery of leukocytes (from FCS fixed blood sample) from each volunteers, error bars represents standard deviations (SD) from their respective means (each volunteer with triplicate sampling). The relative recovery of total leukocytes was found in the range of 44–66% with a mean of  $51.4 \pm 7.4$ . Mean of the recovered neutrophils, lymphocytes and mixed cells (monocytes + eosinophils + basophils) (from Day 0 to Day 120) were  $54.5 \pm 6.1$ ,  $50.7 \pm 9.4$  and  $49.2 \pm 8.1$  respectively.



**Figure 3. Recovery of leukocytes from FCS-fixed whole blood samples at different days of incubation**

Relative recovery of leukocytes from fixed blood sample at day 0, 5, 30, 60, 90 and 120, from (A) 5 individuals (cumulative data), error bars represent Standard Error of Mean (SEM) ( $n=5 \times 3$  i.e., 5 individuals with triplicate sampling), (B–F) Five discrete/independent volunteers, data represented as mean  $\pm$  standard deviation (SD), ( $n=1 \times 3$ , i.e., each volunteer with triplicate sampling).  $>6000$  cells were analyzed per individual per incubation time. FCS fixed, neutrophils, lymphocytes, and mix cells (monocytes + eosinophils + basophils) were compared with control sample, obtained by RBC lysis method from same group of volunteers for estimation of percent relative recoveries. All the statistical analysis and curve plotting was carried out using software Origin 2018 (9.5).

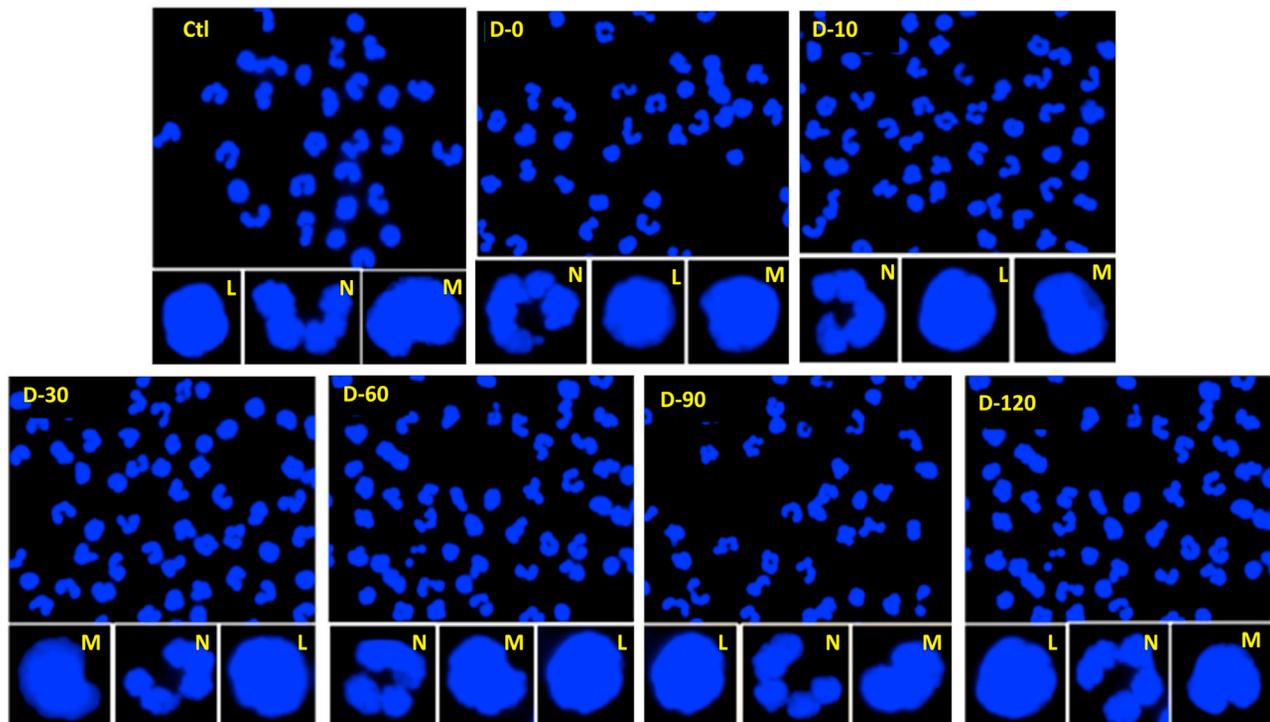
Morphological examinations of the recovered leukocytes from various time points were made by fluorescence microscopy. It was observed that, shape, size, morphology, and integrity of nuclei (of all kinds of leukocytes) were intact/unchanged (Figure 4). Neutrophils can be identified with their characteristic 3–5 lobed nucleus, joined by slender strands of genetic material (Chan et al., 2010; Nothan, 2006). Lymphocytes and monocytes can be identified by their characteristic round (uniform) and indented (kidney shaped) nuclei, respectively (Jacob, 2016; Sarrafzadeh et al., 2014). It is evident from the field view (Figure 4), neutrophils were the most abundant followed by lymphocytes, and other cells (monocytes, eosinophils and basophils) were least in counts. It was observed that, integrity of the cytoplasm was not maintained/preserved in the tested proportion of blood and FCS, described above.

### Long-term preservation of chromatin regions in fixed leukocytes, demonstrated by G0-FISH

To demonstrate intactness of the chromatin regions, in fixed leukocytes, G0-fluorescence in situ hybridization (FISH) was performed, with centromeric probes (Metasystems, Altusheim, Germany), following the manufacturer's guideline with slight modifications (Ly et al., 2017). As, illustrated in Figure 5, centromeric regions of chromosome pair 1, were intact in their territories. Similar observations were found with centromeric probes of chromosome Y (figure not shown here). These results indicate that, chromatin regions were intact in their territories, in fixed leukocytes, which can be explored for detection of any locus specific changes in the chromatin for various clinical and other applications.

### Long-term preservation of radiation-induced chromosomal translocations, fragmentation, and apoptosis in fixed leukocytes

To evaluate fixation abilities of FCS for radiation induced changes in the chromatin, blood samples were irradiated with 2 Gy of  $^{60}\text{Co}$ - $\gamma$ -rays in Blood Irradiator-2000 (manufactured and supplied by



**Figure 4. Field view showing morphological characteristics of leukocyte nuclei recovered from fixed blood samples, at day 0, 10, 30, 60, 90, 120 (stained with nuclear dye DAPI)**

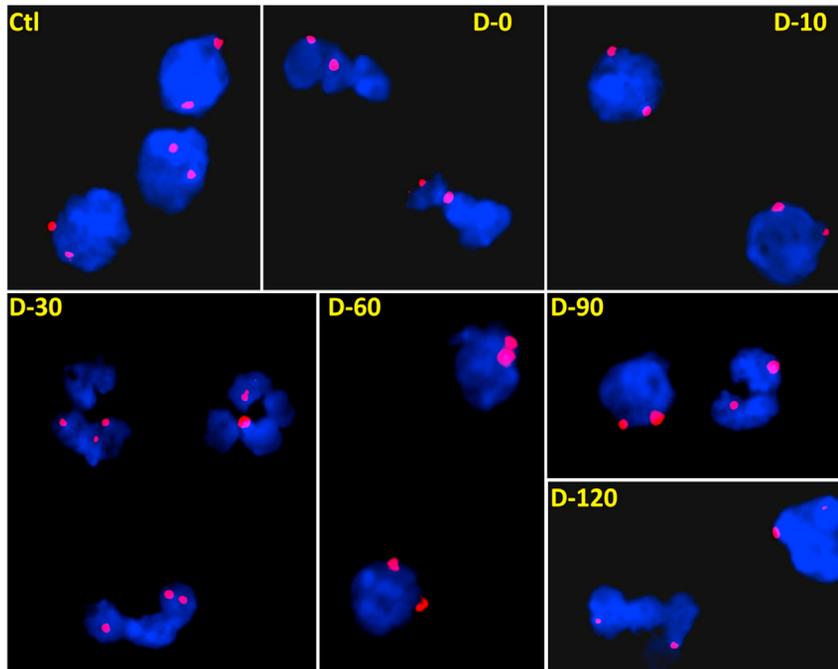
Leukocytes harvested by RBC lysis method from unfixed blood sample of the same individuals were taken as reference/control. Abbreviations M, N and L stands for monocytes, neutrophils, and lymphocytes respectively.

Board of Radiation & Isotope Technology (BRIT), DAE, India) and incubated (in optimum conditions) for 4 h, before fixation, to allow DNA repair to occur. G0-FISH was performed for chromosome pair 1 (with whole chromosome paint probe, from Metasystems, Altlussheim, Germany) following manufacturer's guideline with slight modifications (Barquintero et al., 2017; Chaurasia et al., 2021). As illustrated in Figure 6B, chromatin fragments and or translocations were found preserved/intact in the fixed leukocytes. Apoptotic cells i.e., cells with nuclear and chromosomal fragmentations and blebbings (He et al., 2009) were distinguishable/identifiable (Figure 6C) in fixed leukocytes. No unexpected changes in the irradiated or control (Figure 6A) leukocytes were observed due to the process of fixation. Apoptosis and chromosomal fragmentation/translocation, are established marker of, not only radiation exposure but many other physical, chemical and biological stresses to the cell (Grégoire et al., 2018; Payne et al., 1992), which opens up a wide window for the practical application/utility of FCS.

## LIMITATIONS

Although nuclear morphology and integrity are maintained well even after 4 months of incubation at ambient temperature after fixation in all the leukocytes, G0-FISH results had shown little flary edges of the hybridized regions of the chromatin after around 3 months of fixation and storage at ambient temperature. However, this can easily be rectified during imaging by adjusting the parameters of gain and the lower and upper thresholds in the software.

Preservation of proteomic antigens/markers such as  $\gamma$ H2AX, 53BP1 and ATM, in the fixed WBCs, were tested, by immunofluorescence assays with fluorescence microscopy and flow cytometry. It was observed that these antigens/markers were not preserved in the fixed WBCs, recovered at all incubation time points, mentioned above.



**Figure 5. G0-FISH, with centromere-specific probe (red) for chromosome pair 1, in fixed leukocytes recovered at day 0, 10, 30, 60, 90 and 120**

Centromeric region of chromatin-1 remains intact up to a period of 120 days after fixation. Leukocytes harvested by RBC lysis method from unfixed blood sample was taken as control.

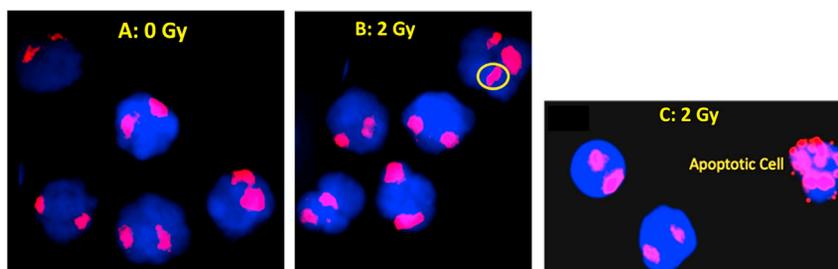
## TROUBLESHOOTING

### Problem 1

What should be done if the pellet of RBCs and platelets is not getting dissolved after 2–3 washings (step 7, in [step-by-step method details](#)) with stock FCS solution (Mixture 1).

### Potential solution

The number of washings can be increased to 3–5 (no changes were observed in the nuclear morphology and genomic integrity of preserved WBCs up to 5 washes), to dissolve most of the debris of RBCs and platelets. If contamination of debris persists, a small increase in the proportion of glacial acetic acid in FCS recovery cocktail will resolve the issue; 10–30  $\mu\text{L}$  of glacial acetic acid can



**Figure 6. G0-FISH in fixed leukocytes recovered at day 20, exposed with 0 (unirradiated) and 2 Gy of  $^{60}\text{Co}$ - $\gamma$ -rays prior to fixation**

First pair of whole chromosomes is hybridized with red probes. No chromosomal translocations/fragmentation were observed in the 0 Gy control sample (panel A); a translocation/fragmentation can be seen in 2 Gy sample (panel B) and marker of apoptosis (programmed cell death) i.e., chromosomal fragmentation and blebbing can be seen in the 2 Gy G0-FISH processed slide (panel C).

be increased in 10 mL of FCS stock solution (Mixture 1), step by step increase of 10  $\mu$ L, at the max 30  $\mu$ L (in 3 steps). Time of treatment was kept same, as described earlier. No change in the nuclear morphology and genomic integrity of WBCs, were observed up to 30  $\mu$ L extra added glacial acetic acid, per 10 mL of FCS stock solution (Mixture 1).

**Note:** Increase in the proportion of glacial acetic acid can also affect the integrity of the membrane of WBCs, therefore it is recommended to increase it gradually in a well-controlled manner, as described above.

### Problem 2

What should be done if integrity of the membrane is not well preserved after fixation ([expected outcomes](#) section).

### Potential solution

A little gradual decrease (10–30  $\mu$ L of glacial acetic acid can be decreased/reduced, per 10 mL of FCS stock solution i.e., Mixture 1) in the proportion of acetic acid can be tried, as acetic acid is responsible for membrane destabilization.

**Note:** FCS can be used for the preservation of various other kinds of cell lines too. However, the proportion of glacial acetic acid may have to be slightly increased or decreased, depending on the cell line, as the composition of membrane varies with the nature of cell types/lines.

### Problem 3

What should be done if nuclear morphology of the recovered WBCs shows physical damages ([expected outcomes](#) section)?

### Potential solution

It is recommended to be more careful about the selection/use of the micropipette while re-suspending cell pellet at the final step of the cell recovery. It is advisable to use tips with wide opening, such as tips used in 20–200  $\mu$ L or 100–1000  $\mu$ L micropipettes to avoid mechanical shear, which may lead to physical injury to the nuclear membrane of the preserved WBCs.

### Problem 4

What should be done if pH of the working FCS is falling out of the described range ([materials and equipment](#) section, Mixture 3)?

### Potential solution

If all constituents are added/mixed properly as described above, pH of the working FCS will fall in the range of 2.3–3.1. However, if pH is falling out of the described range, it is recommended, not to adjust pH using any acid or alkali, prepare fresh working FCS solution, discard older one.

### Problem 5

How can low temperature stored (4°C) blood sample be fixed with FCS, is the procedure same as for the fixation of fresh blood (Step 1, in [step-by-step method details](#))?

### Potential solution

Allow low temperature (4°C) stored blood sample (maximum stored up to 24 h) to approach to the room temperature (RT). Then, mix it well either with the help of 1 mL micropipette or by gently inverting the blood containing tube (8–10 times) manually. Once RT is achieved, blood sample can be poured drop by drop in the FCS tube with gentle shake, as described above.

### Problem 6

How often should the user check the pH of blank FCS ([materials and equipment](#) section, Mixture 3)?

### Potential solution

If, blank FCS is stored in an airtight container, pH need not be checked on a regular basis, as on opening of the tube, pH goes up owing to evaporation of glacial acetic acid, which can be avoided. pH should be checked just before the use of the FCS vials; if pH is falling out of the recommended range, these vials should be discarded and fresh vials need to be prepared.

### RESOURCE AVAILABILITY

#### Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contacts, Dr. Balvinder K. Sapra ([bsapra@barc.gov.in](mailto:bsapra@barc.gov.in)).

#### Materials availability

This study did not generate new unique reagents.

#### Data and code availability

This study did not generate a new dataset or code for analysis.

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### AUTHOR CONTRIBUTIONS

Conceptualization, R.K.C. and B.K.S.; methodology, R.K.C., B.K.S., and K.B.S.; investigation, R.K.C., B.K.S., and K.B.S.; writing-original draft, R.K.C. and B.K.S.; writing-review & editing, R.K.C. and B.K.S.; and funding acquisition, this work was financially supported by host institution (Bhabha Atomic Research Centre, Mumbai, India); no external funding was involved.

### DECLARATION OF INTERESTS

The authors declare no competing interests.

### REFERENCES

- Barquintero, J.F., Beinke, C., Borràs, M., Buraczewska, I., Darroudi, F., Gregoire, E., Hristova, R., Kulka, U., Lindholm, C., Moreno, M., et al. (2017). RENEb biodosimetry intercomparison analyzing translocations by FISH. *Int. J. Radiat. Biol.* 93, 30–35.
- Chaurasia, R.K., Bhat, N.N., Gaur, N., Shirsath, K.B., Desai, U.N., and Sapra, B.K. (2021). Establishment and multiparametric-cytogenetic validation of 60 Co-gamma-ray induced, Phospho-gamma-H2AX calibration curve for rapid biodosimetry and triage management during radiological emergencies. *Mutat. Res. Genet. Toxicol. Environ. Mutagen.* 866, 503354.
- Chan, Y.K., Tsai, M.H., Huang, D.C., Zheng, Z.H., and Hung, K.D. (2010). Leukocyte nucleus segmentation and nucleus lobe counting. *BMC Bioinformatics* 11, 1–18.
- de Resende, M.M., Stodola, T.J., and Greene, A.S. (2010). Role of the renin angiotensin system on bone marrow-derived stem cell function and its impact on skeletal muscle angiogenesis. *Physiol. Genomics* 42, 437–444.
- Grégoire, E., Roy, L., Buard, V., Delbos, M., Durand, V., Martin-Bodiot, C., Voisin, P., Sorokine-Durm, I., Vaurijoux, A., Voisin, P., et al. (2018). Twenty years of FISH-based translocation analysis for retrospective ionizing radiation biodosimetry. *Int. J. Radiat. Biol.* 94, 248–258.
- He, B., Lu, N., and Zhou, Z. (2009). Cellular and nuclear degradation during apoptosis. *Curr. Opin. Cell Biol.* 21, 900–912.
- Jacob, E.A. (2016). Complete blood cell count and peripheral blood film, its significant in laboratory medicine: a review study. *Am. J. Lab. Med.* 1, 34.
- Ly, P., Teitz, L.S., Kim, D.H., Shoshani, O., Skaletsky, H., Fachinetti, D., Page, D.C., and Cleveland, D.W. (2017). Selective Y centromere inactivation triggers chromosome shattering in micronuclei and repair by non-homologous end joining. *Nat. Cell Biol.* 19, 68–75.
- Nothan, C. (2006). Neutrophils and immunity: challenges and port unities. *Nat. Rev. Immunol.* 6, 173.
- Payne, C.M., Bjore, C.G., Jr., and Schultz, D.A. (1992). Change in the frequency of apoptosis after low-and high-dose X-irradiation of human lymphocytes. *J. Leukoc. Biol.* 52, 433–440.
- Sarrafzadeh, O., Rabbani, H., Talebi, A., and Banaem, H.U. (2014). Selection of the best features for leukocytes classification in blood smear microscopic images, 9041 (International Society for Optics and Photonics), p. 90410P, <https://doi.org/10.1117/12.2043605>.