CHAPTER 3

Methodology

Outline

The overall outline of the research methodology is on Figure 9. The procedures 3.1 and 3.2 are a standalone experiment that deals with the field study of the mining area and the laboratory experiment to induce aberration in the lab specimen respectively. While 3.3 is the methodology of the preparation of the cells for observation.

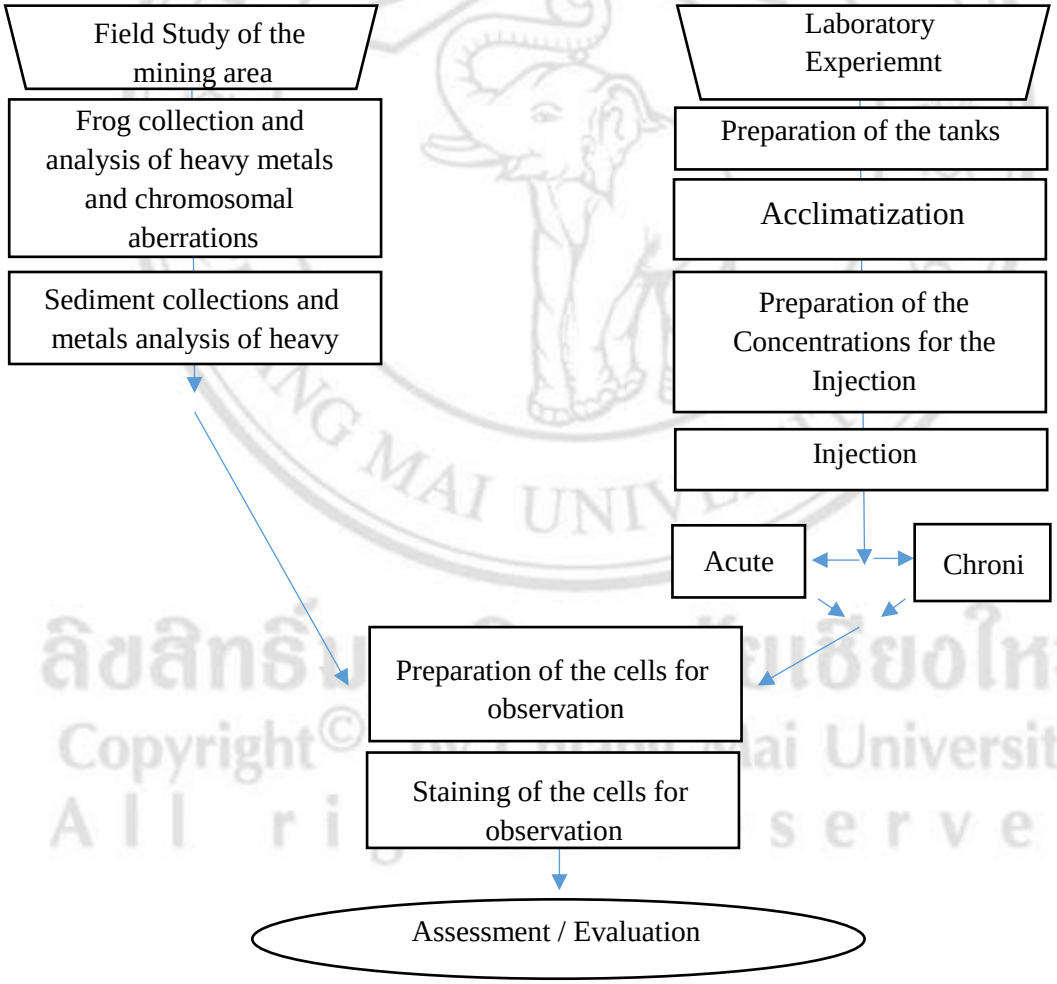

Figure 9 the outline of the methodology.

3.1. Field Study of the Mining Area

3.1.1. Study Site

The study area is in Pichit Province (Figure 10), 330 km North of Bangkok, Thailand. The main gold deposit of the area is from the Loei-Phetchabun-Ko Chang volcanic belt (Vivatpinyo, Charusiri, and Sutthirat 2014). There are three study sites. Two mines and one reference site 6.75 km north of the study sites (a condition by the funding agency). The reference site (Figure 11) is a roadside rice farm plot of approximately 3960 m^2.

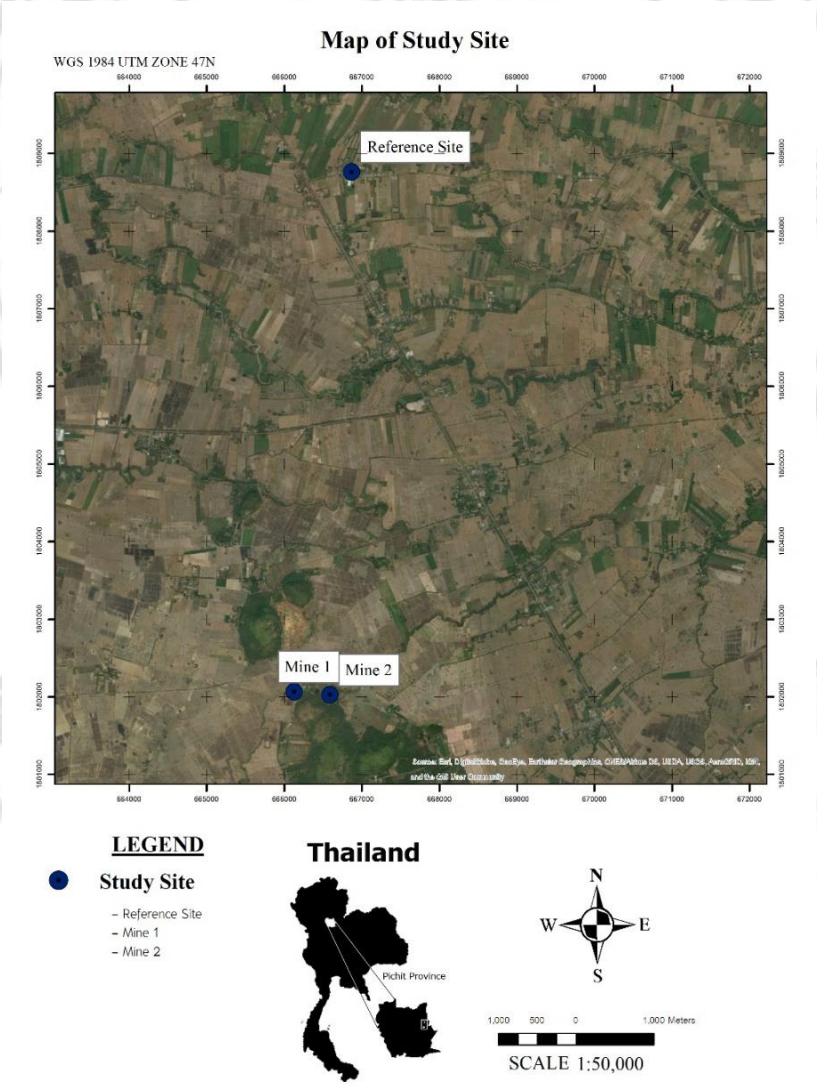

Figure 10 the study sites are located in Pichit Province, Thailand.

Figure 11 reference site, a farm with no mining activity about 6.5 Km north from the mines.

Figure 12 mine 1 (Site 1) fish farming pool.

Mine 1 (Site 1) (Figure 12) is a fish farming pool which doubles as a mine water dump of approximately 21300 m^2 next to a well vegetated road. There is unlimited access to the mobile sediment collectors in every direction of the area. The area is heavily used.

Figure 13 mine 2 (Site2), a farm with different types of crops.

Mine 2 (Site 2) (Figure 13) is a section in a farm of approximately 9800 m^2 specially designed for housing of 4 permanent sediment collectors. The wastewaters drained to larger eutrophic pools at the back, is used for irrigation, and shrimp farming. These mines, because of the mode of mining practiced with the combination of farming, becomes ideal areas of research to explore contamination and human exposure.

3.1.1. Sediment Collection and Analysis

Sediment were collected following the Cornell Framework (2016). The representative samples were collected using a “W” walk trail (Figure 14). Sub sampling was carried out into a bucket taking two trowels of even size. The samples from the site were mixed (Figure 15) in the bucket and stored in small sample bag of about 3 Kg.

All the sediment were collected using clean plastic apparatus and was stored in clean plastic bags at room temperature during the transportation for analysis. The samples were

checked for any algal, plant matter or bio matter during this collection, as this will influence the results.

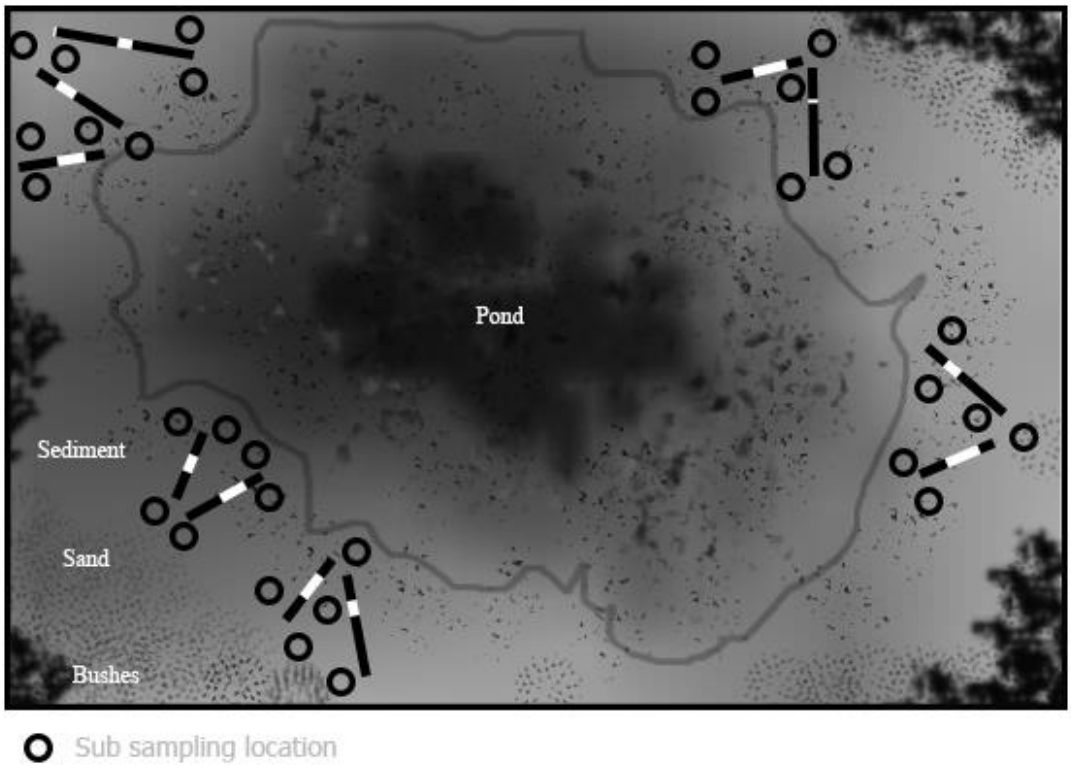

Figure 14 a diagrammatic example of sub sampling process according to the Cornell Framework.

The sediment samples were digested using the EPA 3052 (USEPA SW-846 Test Method 3052: Microwave Assisted Acid Digestion of Siliceous and Organically Based Matrices 2015) method. The samples were homogenized and weighed to the nearest 0.001 g in a vessel, which was equipped with a pressure relief mechanism. This followed the addition of concentrated HNO_3 and concentrated HF to the sample in a fume cupboard. Finally, the samples were microwaved and heated for an appropriate time interval.

3.1.2. Frog Collection and Analysis.

The sampling for the frogs was carried out at night, as the frogs are nocturnal. Head mount flashlights helped to track the movement of the fleeing frogs, and the reflection of the

eyes of the cornered frogs helped locate them. From each location, a minimum of four specimens of *Fejervarya limnocharis* and *Fejervarya cancrivora* were caught. The frogs were handled gently to minimize the sampling damage and stress.

The frogs were put into a closed system enclosures prepared prior to sampling using vegetation from the sampling site as a habitat following the works of Poole and Grow S, (2012). The vegetation added minimized the stress of the transfer. The vegetation used for each container was specific for the study site assuming that the vegetation will contain study site-specific pollutants. After the transfer, a little bit of the water from the study site completed the habitat.

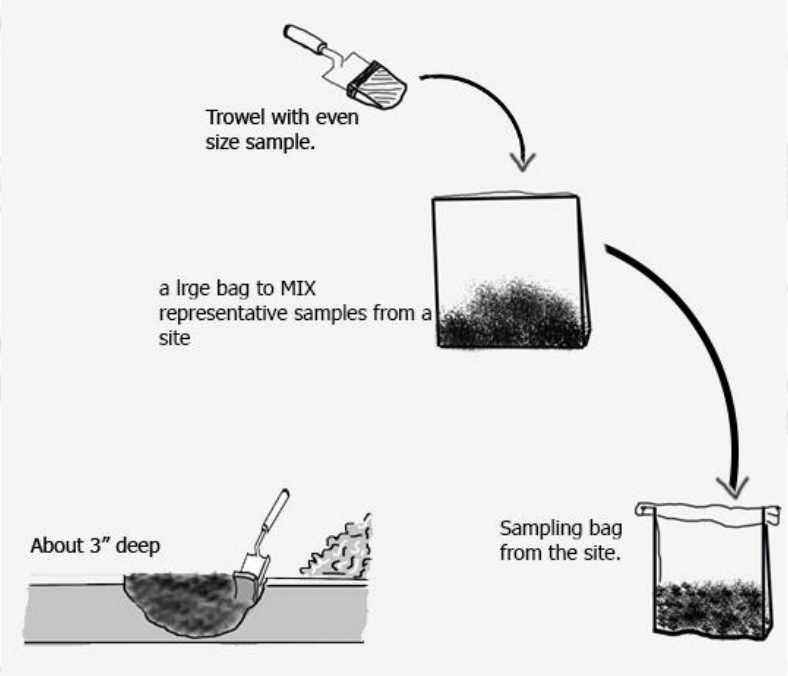

Figure 15 A diagrammatic example of mixing the sub samples for a representative sample.

After transfer to the laboratory, the habitats were covered with dark paper to reduce stress. The cover wrapped all around the habitat leaving the top to allow ventilation. The top of the containers had a plastic net mesh that fits well to the top to prevent the loss of samples. Two specimens of both species were frozen as soon as possible to send for the analysis of the metals.

Frozen samples of the frogs were digested using the AOAC official method of 2013 (Julshamn et al. 2013) where, the sample was homogenized using cleaned apparatus. The homogenized samples were dehydrated depending on the moisture content and drying factor either in an oven or over $Mg(CIO_4)^2$ in a desiccator until constant mass was reached. Then the samples were digested in a sealed pressure digestion vessel using HNO_3 and H_2O_2 followed by the microwave heating. The test solution obtained after digestion was diluted and tested against reference material using an ICP-MS model 7500C.

3.2. Laboratory Experiment.

3.2.1. Preparation of the Tanks

Glass tanks of dimensions 65 X 31 cm and 52 cm deep with reinforced top and bottom was used as the frog's enclosures following the works of Poole and Grow S, (2012). Initially the closed system enclosures were cleaned, and dried in the sun. This followed the introduction of the habitat. Followed by a "heat-drying" using bulbs overnight at 30 ̊C. After which they were washed with filtered water and kept overnight at 30 ̊C. This followed a complete draining and re- filling of the tanks with water to the required level.

3.2.2. Acclimatization

The frogs from the transport cage were washed with filtered water removing any foreign material on the skin. This followed the sorting of the frogs into their weight classes. The frogs were observed for aggression, mating behaviour, and feeding. Which allowed further refining of the grouping. The frogs were fed commercial frog feed CP 9921 which contains protein >25%, Fat > 3%, Moisture < 12% and other ingredients namely fish meal, soya bean meal, rum, coconut meal, soya bean steamed, maize rice germ, vitamins, nutrients and preservatives which makes up to <8%.

The tank were cleaned at least twice every day removing all frog waste replacing the water with fresh filtered water. The tanks were kept at a constant 30 ̊C using two 60 W yellow lights for 12 hours every day. The frogs were given the same treatment for 10 days.

3.2.3. Preparation of the Concentrations for the Injection.

The average concentration in mg/kg can be converted to mg/ml directly as the substances will be dissolved in water where the density is 1 kg/l. We did consider administering a dosage from the prepared standard solution by calculating the dosage. However, after much consideration an even volume of different concentrations was decided as the best practice for this experiment as this ensures that the control will get its injection.

Using a study by Suttichaya et al. (2016) that analysed the chromosomal aberrations of the species *Hoplobatrachus rugulosus* from a Gold mine with arsenic contamination. The tissue concentrations in their study are 0.708, 0.349, and 0.121 mg/kg. This caused a positive chromosomal aberration on all individuals. Hence, the injections are set using this study as a baseline.

The injections were derived from a primary concentration made by dissolving 104 mg of $Na_2HAsO_4.7H_2O$ in 250ml deionised water to make 100 mg/l as per the laboratory protocols in applied life sciences by Bisen, (2014). A secondary standard of was made to 3 mg/l in 250 ml standard flasks by further dilutions. The injections of concentrations were made from 00.00, 00.50, 01.00, 01.50, and 02.00 mg/l using the secondary standard concentration diluted in a 25 ml standard flasks. The frogs were injected to the dorsal side of the frogs.

3.2.4. Injection of the Frogs

The acute and chronic exposure has been defined as a single large dose and small repeated doses respectively (Britannica 2018). The methodology is derived from the studies of Krishnaja and Rege (1982) adopted for amphibians.

Acute exposure is generally limited to a single dose observed for 5 to 6 days. (Ali et al. 2008). For acute exposure, $Na_2HAsO_4.7H_2O$, injections of concentrations 00.50, 01.00, 01.50, until 02.00 mg/l were administered with a control. The frogs were injected once and observed for 4 days. Two frogs were used per concentration.

Chronic exposure usually is of multiple doses ranging from 10 - 20 days (Fort et al. 2004; Navarro-Martín et al. 2014; Olausson et al. 2013; Peles 2013). For chronic exposure, $Na_2HAsO_4.7H_2O$, injections of concentrations 01.00, 01.50, until 02.00 mg/l were administered with a control. The frogs were injected every 4 days until 20 days. Two trials were used per concentration with three frogs per trial. The injections were administered while the frogs were held upside down to ensure the shift of the organs up into the cavities to ensure peritoneal injection.

3.3. Preparing the Cells for Observation

The cells were observed from the bone marrow of the frog. The frogs were prepared according to the methods of Chen and Eeling (1968) and Nanda et al.(1995). 0.1 ml of Colchicine ($C_{22}H_{25}NO_6$) per every 10 g of frog tissue was administered through inter peritoneal injections to the dorsal side of the frog and kept under observation for 8 hours. The frogs were transferred to a freezing refrigerator to slow down the metabolism and to kill them. Once they are immobile, the femur and the tibiofibula were removed. The epiphysis was cut off from the top and the bone marrow removed from the bone using a sterile injection in to a sterile cell culture dish with 0.075M KCl and crushed. The crushed bone fluid in the 0.075M KCl was transferred to a centrifuge tube. The bones in the dish was washed with 0.075m KCl using a pastor pipette and the fluids were transferred to the centrifuge tube till the liquid was 7 ml. The tubes were incubated for 20 minutes. After the incubation, the tubes were centrifuged at 2000-25000 rpm for 10 minutes. The supernatant was discarded using an assigned specific pipette. This was followed by the drop wise addition of fixative (3 methanol: 1 glacial acetic acid) and further centrifugation of the tube at 2000-2500 rpm at 10 minutes. This was repeated until the supernatant became clear.

3.4. Staining of the Cells for Observation

3.4.1. Preparation of the Slide

Slides from each sample was cleaned and labelled accordingly. In a fume cupboard, all of the slides were cleaned using a drop of fixative (3 methanol: 1 glacial acetic acid), and sterile wipes.

3.4.2. Preparation of the Samples

Before the samples could be added to the slides, the samples were centrifuged at 2000 — 2500 rpm for 5 minutes. Followed by the removal of the supernatant without disturbing the precipitate.

3.4.3. Fixing of the Samples on the Slide

The cells were fixed on the slide by gently drawing about 0.5 ml from the top of the pellet using a pastor pipette and dropping 2 drops on to the slide and allowing them to dry.

3.4.4. Staining with Giemsa's stain

Giemsa's stain was prepared by adding 10 ml of Giemsa's stain to 90 ml of buffer solution. The dried slides were immersed in Giemsa's stain for 20 minutes (Rooney 2001). After 20 minutes, they were dipped in water for about 5 minutes taken out and air dried.

3.5. Observation

3.5.1. Chromosome analysis

Cytogenetic analysis and recording was performed on mitotic metaphase cells under a light microscope fitted with a Canon EOS 700 D digital camera using an Olympus Om system adopter. The analysis was done using a computer connected through an HDMI cable to a high definition Television.

2.5.2. Identification of Aberrations

According to the Genetic Alliance (2009) all chromosomal abnormalities can be categorised as either numerical or structural. A numerical abnormality is when; a whole chromosome is either missing or is extra to the normal pair. While, a structural abnormality is; when a part of an individual chromosome is missing, extra, switched to another chromosome, or turned upside down. Structural abnormalities are due to breakage and incorrect re-joining of chromosomal segments. In a structural rearrangement, if the complete chromosomal set is still present although rearranged it is. Balanced rearrangements include inverted or translocated chromosomal regions. Since the full complement of DNA material is still present, balanced chromosomal rearrangements may go undetected. A structural rearrangements is unbalanced if information is additional or missing. The aberrations of concern in detail are as follows.

i) Breaks

A break (Figure 16) is when; the chromosome breaks due to an external factor and joins in various ways resulting in aberrations.

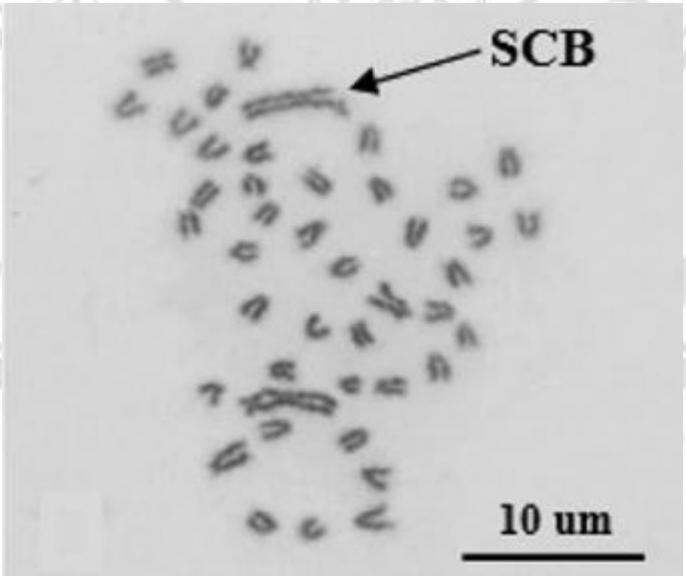

Figure 16 single chromatid break observed by Tengjaroenkul et al, (2017).

The resultant aberrations can be observed in the next metaphase stage (Natarajan 1984). The pollutant or agent will damage the DNA sequence. This then goes through a synthesis, where they may or may not go through repair (Hittelman 1990). Chromatid breaks are breaks in chromatids with the broken part more or less dislocated from the chromatids from which they descend (Recklinghausen et al. 2007). Breaks result in fragments (Figure 17) and sever fragmentation causes minute chromosomes (Preetpal and Tripathi 2014)

a. Double Strand Breaks

DNA double-strand breaks (DSB) are the most deleterious lesions directly or indirectly induced by DNA-damaging agents, and are occasionally formed during DNA replication owing to broken (collapsed) replication forks (Szumiel 2007).

b. Isochromatic Breaks

Isochromatid breaks are when both chromatids of a chromosome are broken at the same site and by this produce fragments without centromeres can also arise from chromosome breaks which occur in un-replicated chromosome regions (Recklinghausen et al. 2007).

ii) Deletions

Chromosome deletions or partial monosomies are in two groups, pure deletions and deletions combined with a duplication, both of which usually occur as the result of reciprocal translocations. Deletions affecting all chromosome ends, as well as many interstitial segments, have been found in live-born children (Grouchy and Turleau 1984; Schinzel 1983). The loss of a chromosome segment is known as a deletion (Sharma 2010). Deletions are also considered as breaks (Savage 1976). These are either terminal or interstitial deletions, they are also labelled as deficiencies (Sharma 2010). Interstitial deletions are very small and mostly appear as dots. Larger acentric rings on separation may show the complex looping and interlocking seen in centric rings. Deletions on a metaphase plate do not remain in the site of origin making it difficult to locate them (Savage 1976). Deletions often lead to ring chromosomes.

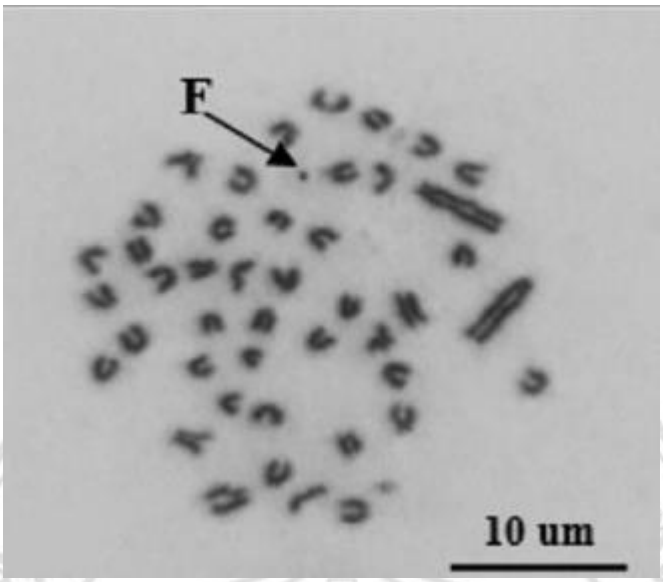

Figure 17 fragments observed by Tengjaroenkul et al, (2017).

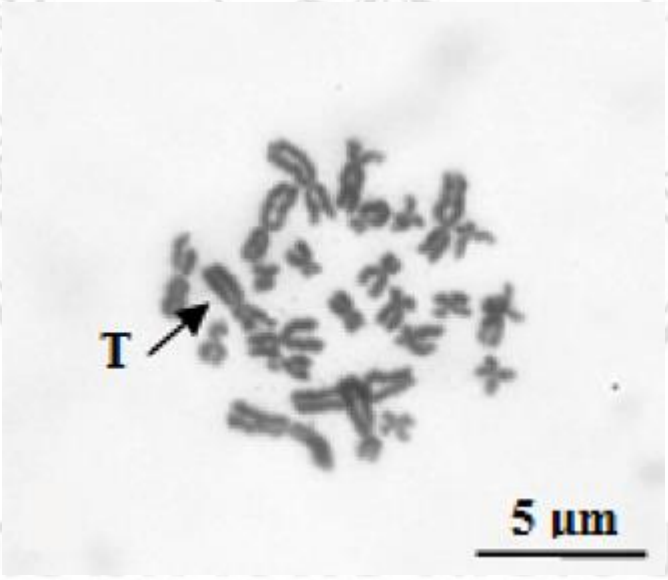

Figure 18 terminal deletion observed by Suttichaiya et al, (2016)

a. Terminal Deletions

Terminal deletions (Figure 18) are deficiencies at the end of the chromosome end (Sharma 2010). However, it is now questionable whether true stable terminal deletions actually exist. All those investigated using the new fluorescent telomere probes were "capped" by telomere sequences. Either means that they are disguised interstitial deletions, where one

re-join point was almost terminal, or that survival is possible by de novo telomere synthesis. The recent development of end-specific telomere probes should be able to solve this question (Boei and Natarajan 1998; Boei et al. 1998)

b. Interstitial Deletions

Interstitial deletions are two breaks, loss of two breaks, and the union of the region of the breaks. Terminal deletions are indistinguishable from interstitial deletions. They appear as a range from very large fragments to very small dots and according to observations smaller deletions are more frequent than larger deletions (Savage 1976).

iii) Gaps

Gaps (Figure 19) are unstained areas in chromatids that are no longer than the diameter of the chromatids in which they occur. This structural change is not usually considered as a real chromosomal aberration. However, they show a similar pattern to chromatid-type chromosomal aberrations (Recklinghausen et al. 2007). Therefore some researchers do not count them as aberrations in their final results (Sram and Rössner 2007).

iv) Ring Chromosomes

Ring chromosomes (Figure 20) appear due to the joining of the terminal ends of the chromosomes forming centric rings and joining acentric fragments forming the acentric rings all of which is possible due to the deletion of telomeric regions. Narrowing of the centromeric region from the sides accompanied by the joining of telomeric ends of sister chromatids was also form rings (Preetpal and Tripathi 2014).

v) Clumping

Extreme stickiness is classified as clumping (Figure 21) and is usually considered as a case of coming together of all or most of the chromosomes of a complement (Preetpal and Tripathi 2014).

vi) Terminal Association of Chromosomes.

Patterns formed due to the erosion of telomeric regions forming a pattern of joining of ends of chromosomes (Preetpal and Tripathi 2014).

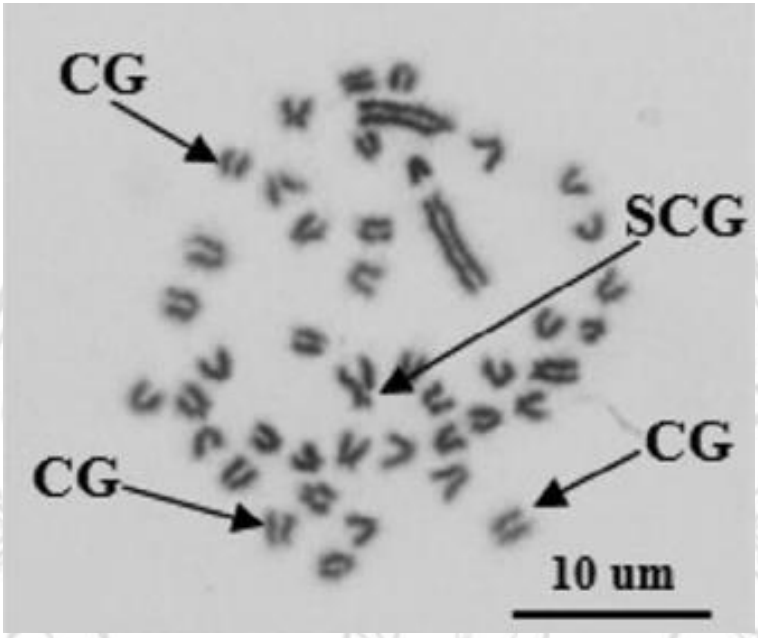

Figure 19 different types of gaps as observed by Tengjaroenkul et al, (2017).

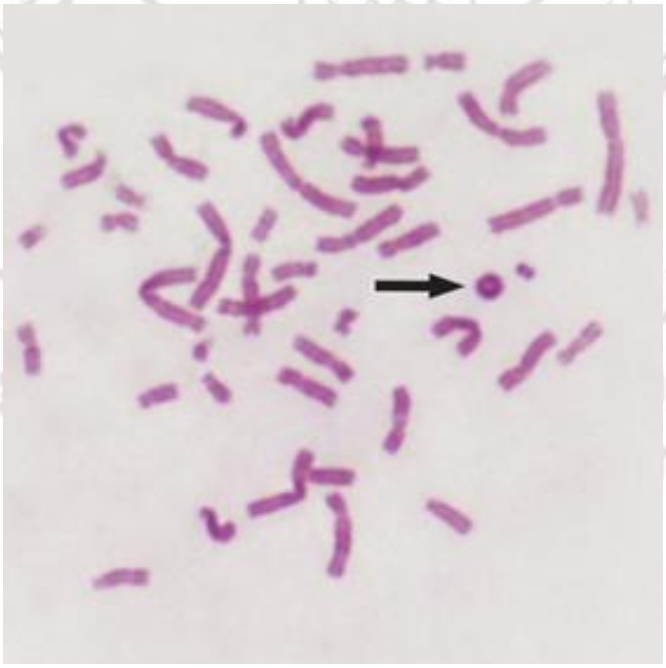

Figure 20 ring chromosomes observed by Zhao et al, (2014).

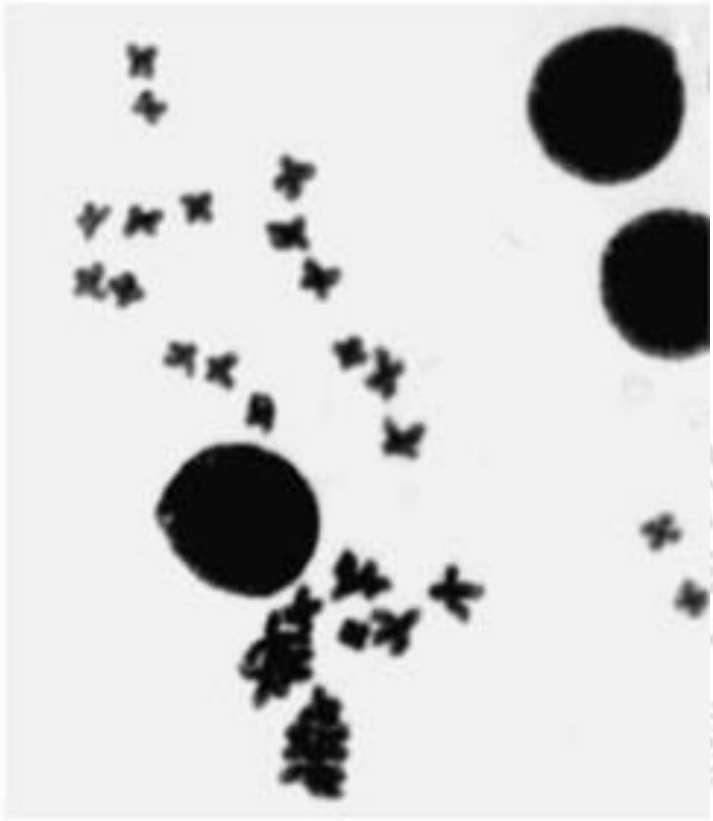

Figure 21 clumping as observed by Zhang et al. (2010)

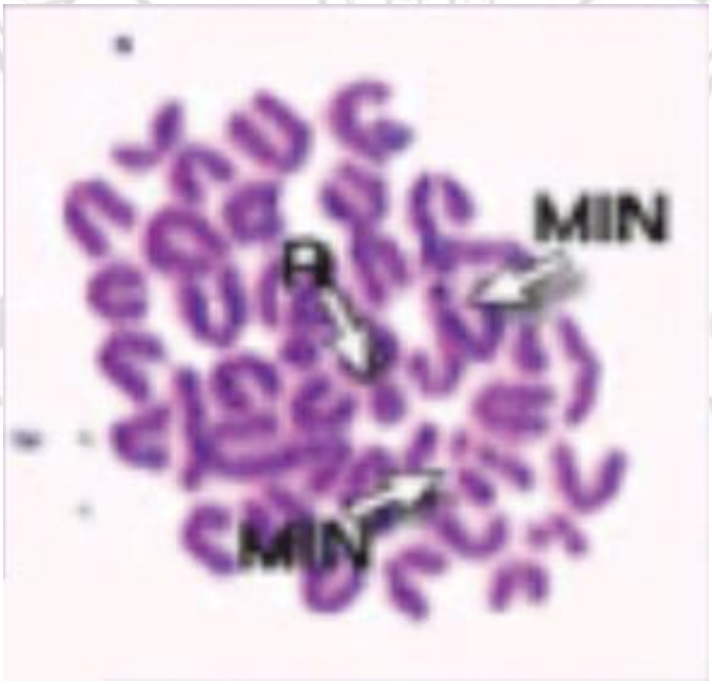

Figure 22 minutes observed by Bansal et al.(2015)

vii) Stickiness

DNA depolymerisation caused the joining of few chromosomes at positions other than their ends is stickiness (Preetpal and Tripathi 2014).

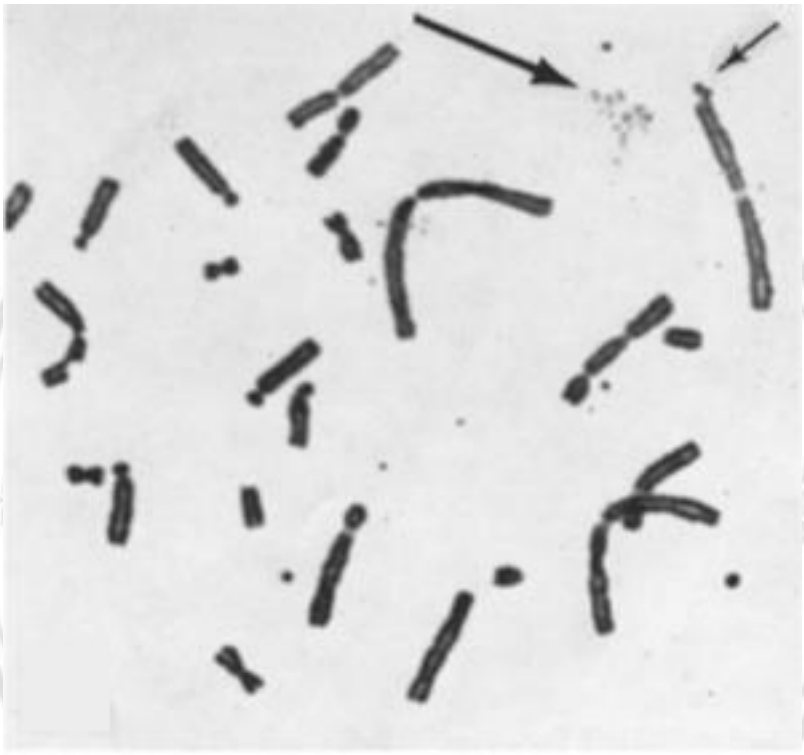

Figure 23 pulverization as observed by Ikeuchi, Weinfeld, and Sandberg (1972).

viii) Minutes

Extreme fragmentation often resulted in minutes (Figure 22). These are smaller than normal fragmentation and often occur in a large number (Preetpal and Tripathi 2014).

ix) Pulverization

Pulverization (Figure 23) is the dissolution of nuclear membrane at interphase , and the formation of nuclear envelopes around metaphase chromosomes (Ikeuchi, Weinfeld, and Sandberg 1972)