Methods for Isolation of High Quality and Quantity of miRNA and Single Cell Suspension for Flow-Cytometry from Breast Cancer Tissue: A Comparative Analysis

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Abstract Inadequate methods may cause substantial loss not only in the quantity but also in quality of the product. This study aimed to determine the best method for making single cell suspension for isolation of RNA and flow cytometer analysis from cancer tissue. We compared two methods of tissue disruption used during RNA isolation and flow cytometer analysis. Mechanical tissue disruption method and enzymatic tissue digestion method are commonly used for making single cell suspension before RNA isolation and flow cytometer analysis. 20 resected tissue samples were dissociated into single cells by mechanical and enzymatic methods. Quality and quantity of isolated miRNA was graded by the ratio of 260/280 nm and by running gels. The results revealed that mechanical hand held tissue homogenizer showed better yield than enzymatic (719.12 ± 513.67 vs. 524.87 ± 388.18 ng/ μ l) and the quality 260/280 nm ratio was significantly better [2.15 ± 0.21 vs. 1.57 ± 0.23 ; 95% CI (0.402–0.730); $p < 0.001$] in mechanical method than enzymatic. However, for flow cytometer enzymatic digestion was best. The mechanical method is very suitable for isolating miRNA than enzymatic while enzymatic digestion is most

favorable for flow-cytometer analysis as it reduces debris in comparison of mechanical process of shearing.

Keywords RNA isolation · Flow cytometer · Mechanical and enzymatic tissue disruption · Quality and quantity · Debris

Introduction

Exploration of effective method for making cell suspension that could be used in isolation of high-quality RNA or flow cytometer analysis from restricted amount of human origin tissue samples is very difficult. As we are familiar that tissue obtained during gun-biopsy per core is not more than 100 mg so to utilize this less amount of tissue in RNA isolation and for characterizing diverse antibodies in flow cytometer, is the key challenge for molecular biologists.

Relentless progress of molecular biology and molecular techniques in last 30 years has now opened new vistas in study of Omics like genomics, transcriptomics and proteomics that could be utilized in solving the puzzle of chronic and untreatable diseases [1–3]. For such diverse branches generally we require fundamental molecules like DNA, RNA or proteins, these molecules reside in the tissue, cells, cell membranes, which enclose their cytoplasm and genomic contents. These molecules have shown potency to be used not only as biomarkers but also in tracing various disease associated pathways [4–7]. Harsh sample treatment could affect nucleic acid quality, quantity along with many debris which may affect further gene expression, flow cytometer analysis. Therefore, it is important to optimize the tissue disruption methods to obtain RNA, or single cell suspension from plentiful as

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well as from minute tissue representatives obtained during surgery or during core biopsy.

Cell disruption cannot be deliberated as an isolated process, as it influences the physical properties of the cell slurry, thus indirectly affecting further downstream processes. Numerous types of cell disruption methods exist, as biological samples may be extracellular, intracellular or periplasmic. Cell disruption methods can be classified into mechanical methods and non-mechanical methods. Mechanical methods are based on solid shearing or liquid shearing methods by using sonication, bead mill and homogenizer. For small tissue < 2000 mg generally we focused on hand held homogenizer having 3000–8000 rpm. Non-mechanical methods can be divided into physical methods (Thermolysis), chemical methods (Detergents, Chaotropic agents) and enzymatic methods. In enzymatic digestion, at least one protease (for example, trypsin or collagenase), cellulose or lipase, or a mixture thereof, can be used as a solution that interacts with the tissue and dissociates it.

Therefore, in current study we compared the mechanical method (handheld homogenizer) with enzymatic (Collagenase IV, trypsin) digestion method for breast cancer tissue disruption that could be utilized in downstream processing like gene expression and flow-cytometer analysis.

Materials and Methods

The study was part of standardization of our current cancer project study. The study was initiated after obtaining ethical clearance from the Institutional Ethics Committee of All India Institute of Medical Sciences Jodhpur, India where the study was carried out. The study was conducted over a period of 1 year from June 2016 to 2017. Newly diagnosed and previously untreated 20 patient's breast tissue samples were collected after taking consent as agreed to volunteer for this study. All the patients were between 28 and 80 years of age. 1000 mg of tissue sample was obtained during surgery or core biopsy from each patient and stored in liquid nitrogen till further analysis. All results were analyzed by using appropriate statistical tools like STATS16 and Graph Pad7 (Prism).

Enzymes and Chemicals

Collagenase, Type IV, powder (Thermo Fisher Scientific Cat: 17104019), Trypsin TrypLETM Express Enzyme (1X), phenol red (Thermo Fisher Scientific Cat: 12605010), Phosphate Buffer Saline (Thermo Fisher Scientific Cat 10010023), HBSS (Hank's Balanced Salt Solution: Thermo Fisher Scientific Cat: 14170112).

Instrument

Hand held Homogenizer (Adjustable Speed, Motor Driven Tissue Grinder Cat: GX-3215830).

Protocols and Procedure: Tissue Lysis

Mechanical Tissue Disruption

Whole tissue dissected into 3–4 mm pieces with a sterile scalpel or scissors in saline salt solution (For RNA: PBS solution and for enzymatic HBSS). Placed the tissue in micro centrifuge tubes of 2.0 ml with PBS or HBSS. The handheld homogenizers speed should be increased from 3000 rounds per minute to 8000 rounds per minutes by providing time interval of 3 min at each speed and 20–25 s halt to prevent overheating of the solution. Then RNA isolation protocols was started as per manufacturer's recommendation (RNeasy Mini Kit: Qiagen), similarly the cell suspension were stained for Flow cytometer analysis (BD Biosciences Kit: Antibodies kit for Serotonin with corresponding Isotype).

Enzymatic Tissue Disruption

Trypsin Based Digestion

Complete piece of tissue is chopped into 3–4 mm pieces with a sterile scalpel or scissors. Wash the tissue pieces by resuspending in a balanced salt solution. The whole process of washing was repeated by 2 or 3 times. Then container with the tissue pieces was transferred on ice. Further, we added 0.25% trypsin in a balanced salt solution without calcium or magnesium (1 ml of trypsin for every 100 mg of tissue) and incubated at 4 °C for 6–18 h to maximize penetration of the enzyme with little trypsin activity. After incubation the trypsin solution discarded from tissue. Incubated the tissue pieces with residual trypsin at 37 °C for 20–30 min. Then tissue was dispersed by several time pipetting [8]. Then filtered the cell suspension by cell strainer (100 and 60 micron: corning). Then RNA isolation (RNeasy Mini Kit: Qiagen), and flow cytometer needed staining (BD Biosciences Kit: Antibodies kit for CD44 & CD24 with corresponding Isotype) was performed as per standard protocols.

Collagenase Based Digestion

Tissue were dissected into 3–4 mm pieces with a sterile scalpel or scissors. Tissue were washed several times with HBSS. Collagenase (50–200 U/ml in HBSS) were added and the container having tissue incubated at 37 °C for 4–18 h. For increasing dissociation efficiency 3 mM CaCl₂

were added. Then filtered the cell suspension by cell strainer (100 and 60 micron: corning). Then RNA isolation (RNeasy Mini Kit: Qiagen), and flow cytometer needed staining (BD Biosciences Kit: Antibodies kit for CD44 & CD24 with corresponding Isotype) was performed as per standard protocols.

Assessment of the Quality of Isolated miRNA

After isolation of RNA, both the quality and quantity of RNA were assessed by measuring the absorbance at 260 and 280 nm wavelengths on Nano plate reader (Biotek) as shown in Tables 1 and 2. These results were validated by running on 0.8% agarose gel (Figs. 1, 2), and further visualization was done on Gel-Documentation (G Box; Syngene).

Flow Cytometer Analysis: Cellular Debris Aspect

This is an important aspect as dead cells, debris or un-lysed cells create background noise due to its auto fluorescence and thus it influences the peak of pulse during flow cytometer analysis. It is believe that events on a dot plot of forward versus side laser scatter that appear below the lymphocyte cluster in blood analysis or aggregates seen near the origin of both X–Y axis during flow run of any single cell suspension are known as “unlysed cells, dead

cells, or cellular debris.” This debris affects the fluorescence pulse.

The detectors in a Flow cytometer are proficient of picking up signals that lie way below the level of practical value in cell analysis. This background noise (arising from cell debris) can be a substantial nuisance, in that it can severely reduce the number of relevant events (i.e. cells) recorded during acquisition.

Results and Discussion

The achievement of any RNA based analysis hinge on the quantity, purity, and integrity of the RNA [9, 10]. RNA quality is affected not only by the sample's nature but also by the method of tissue lysis or cell suspension is preparation. Thus, our primary objective was to test the performance of two very common method of tissue disruption i.e. by mechanical method and enzymatic method.

As quality of nucleic acids is an important aspect for all transcriptomics, gene expression and RNA editing experiments for its success. It is known that nucleic acids have absorbance maxima at 260 nm. Aromatic amino acids absorb light at 280 nm, so absorbance measurements at that wavelength are used to estimate the amount of protein in the sample. So, the ratio of 260 nm absorbance maximum to the absorbance at 280 nm is used as a measure of purity

Table 1 Showing the quality and concentration of RNA isolated from Breast cancer patients after enzymatic tissue disruption method

Sample read#	260 Raw	280 Raw	320 Raw	260	280	260/280	ng/μL
1	0.176	0.127	0.032	0.129	0.081	1.594	1032.2
2	0.128	0.095	0.036	0.082	0.048	1.692	656.14
3	0.586	0.341	0.034	0.523	0.293	1.788	418.62
4	0.588	0.342	0.034	0.527	0.294	1.79	421.32
5	0.181	0.127	0.041	0.129	0.081	1.594	103.18
6	0.174	0.12	0.034	0.13	0.082	1.587	103.65
7	0.117	0.087	0.035	0.076	0.037	2.06	609.6
8	0.121	0.084	0.033	0.081	0.049	1.659	646.02
9	0.354	0.209	0.032	0.305	0.169	1.797	243.65
10	0.353	0.209	0.032	0.306	0.171	1.793	244.82
11	0.185	0.135	0.034	0.139	0.087	1.598	1120.9
12	0.135	0.12	0.033	0.087	0.072	1.208	696.03
13	0.527	0.362	0.035	0.483	0.316	1.528	386.39
14	0.433	0.38	0.031	0.385	0.334	1.153	308.11
15	0.4	0.327	0.039	0.355	0.281	1.263	284.65
16	0.259	0.153	0.033	0.213	0.107	1.991	1703.9
17	0.764	0.532	0.035	0.714	0.486	1.469	571.1
18	0.481	0.435	0.033	0.431	0.389	1.108	344.79
19	0.413	0.297	0.032	0.368	0.249	1.478	294.4
20	0.432	0.288	0.032	0.386	0.242	1.595	308.8
Mean	0.3408	0.2385	0.034	0.2928	0.194	1.5689	524.87
Std	0.1836	0.1294	0.0024	0.1808	0.1279	0.2289	388.18

Table 2 Showing the quality and concentration of RNA isolated from Breast cancer patients after mechanical method of tissue disruption

Sample read#	260 Raw	280 Raw	320 Raw	260	280	260/280	ng/ μ L
1	0.594	0.342	0.036	0.548	0.294	1.86	438.46
2	0.718	0.361	0.032	0.673	0.313	2.15	539.29
3	0.585	0.295	0.03	0.538	0.249	2.16	430.43
4	0.609	0.302	0.034	0.562	0.254	2.21	449.6
5	0.792	0.399	0.038	0.745	0.352	2.12	596.02
6	0.684	0.358	0.032	0.639	0.31	2.06	511.2
7	0.567	0.267	0.029	0.52	0.219	2.39	416.71
8	0.117	0.078	0.031	0.072	0.0317	2.26	576.09
9	0.095	0.068	0.027	0.0496	0.0218	2.28	396.8
10	0.623	0.314	0.033	0.576	0.268	2.15	460.77
11	0.541	0.275	0.034	0.496	0.227	2.19	396.81
12	0.682	0.353	0.032	0.636	0.306	2.07	508.73
13	0.266	0.145	0.028	0.219	0.097	2.26	1752.2
14	0.28	0.144	0.031	0.244	0.099	2.46	1951.8
15	0.144	0.101	0.03	0.099	0.053	1.88	799.2
16	0.288	0.145	0.033	0.243	0.1	1.99	1944.1
17	0.687	0.398	0.032	0.642	0.351	1.83	513.6
18	0.779	0.336	0.035	0.732	0.29	2.52	585.6
19	0.542	0.251	0.031	0.497	0.203	2.45	397.5
20	0.944	0.552	0.034	0.897	0.503	1.78	717.6
Mean	0.52685	0.2742	0.0321	0.48138	0.227075	2.1535	719.123
Std	0.244072	0.126071	0.002673	0.243183	0.125579	0.213252	513.6716

Fig. 1 Agarose gel showing RNA bands, tissues were disrupted by mechanical method (handheld homogenizer) A and C samples were 2000 mg, others were of 1000 mg weight **Fig. 2** Agarose gel showing RNA bands, tissues were disrupted by enzymatic method, where A, E, H were digested by trypsin and remaining by collagenase, A and C samples were 2000 mg, others were of 1000 mg weight

or quality in RNA extractions. A 260/280 nm ratio of ~ 2.0 is generally accepted as “pure” and high quality for

Fig. 3 Quality assessment of isolated RNA after two different method of tissue disruption

RNA as depicted in Fig. 3. In addition, an absorbance reading at 320 nm can be taken to detect any light-scattering components in the sample like turbidity etc. The 320 nm reading is adjusted from the 260, 280 and 230 nm values as background. The quality of RNA isolated in both the methods (as shown in Tables 1, 2), when compared by using *t* test, it showed significantly ($p < 0.001$) better quality for mechanically disrupted samples than enzymatic method, as mean of 260/280 ratio [$(2.153 \pm 0.213$ vs. 1.568 ± 0.213 ; 95% CI: $0.566(0.402-0.730)$].

Fig. 4 Dot plot diagram of Flow-cytometer showing the difference in debris, first diagram showed mechanical method having more debris than enzymatic method (second diagram on right) of tissue disruption

Similarly, the total RNA yield obtained by these two methods were compared, and it was noticed that yield was comparatively higher in mechanical disruption method than enzymatic (719.123 ± 513.67 vs. 524.87 ± 388.18 ng/ μ l 95%CI: 194 (– 64.9 to 453), although it failed to show any significant difference.

However, enzymatic method of tissue disruption produces less debris or dead cells than mechanical methods as represented in Fig. 4. Debris due to auto fluorescence affects the peak and width of the pulse of fluorescence, thus it may alter actual events recording [9]. Ultimately, dead cells can also seriously affect analysis. These cells are autofluorescent and can lead to increased background through non-specific binding, as well as reduce the dynamic range so that weak positive samples will not be identifiable. These debris cells may hide dim populations resulting in a poor sort. Also, dead cells non-specifically binds antibodies and dyes and result in false positives. Finally, the presence of free DNA (from the dead cells) acts as a “glue” resulting in more cell aggregates and cell

sorter clogs. To remedy this, samples with large amount of dead cells and debris should be cleaned up. Therefore it is highly recommended we should minimize these debris [11]. So as per our results, it is very clear that for RNA or nucleic acid isolation the mechanical methods for tissue disruption are preferable as have shown high quality and purity with yield. However for preparing single cell suspension of flow-cytometer analysis, enzymatic methods are very suitable as they produce less debris than mechanical disruption.

Conclusions

The mechanical tissue disruption method is very suitable for isolating miRNA than enzymatic digestion method as it not only provide better yield or concentration but also a good quality or purity, while enzymatic digestion is most favorable for flow-cytometer analysis as it reduces debris in comparison of mechanical process of shearing.

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Compliance with Ethical Standards

Conflict of interests The authors declare that they have no competing interests.

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