Karl Narvacan, Year 3 Medical Student, University of Alberta
Brain Tumour Foundation of Canada Research Studentship
Supervisors: Dr Frank van Landeghem, Division of Neuropathology and Dr Iyare Izevbaye, Division of Molecular Pathology, University of Alberta

---

Final Report

Title: Evaluation of nCounter technology in copy number variation analysis for the classification, diagnosis and predictive prognosis of diffuse gliomas

Objectives: The purpose of our proposed research project is to evaluate a new analytical method in performing copy number variation (CNV) analysis through the novel nCounter technology to aid in the classification, therapeutic management and prognosis of diffuse brain tumours, including astrocytomas, oligodendrogliomas and glioblastomas. Currently, the gold standard in molecular genetics of brain tumours includes the analysis of *ATRX*, *IDH*, *EGFR*, *PTEN*, 1p/19q co-deletions and *MGMT*, in accordance to the recently published "ISN-Haarlem" guidelines. Fluorescence *In Situ* Hybridization (FISH), the current gold standard technology for analysis of CNVs, suffers from several disadvantages including labor-intensiveness, low CNV resolution, slow throughput and high tissue sample requirements.

Specific Objective: To assess several diagnostic parameters (sensitivity, specificity, positive and negative predictive value and likelihood ratios) of nCounter technology in analyzing copy number variations in neuro-oncology (including 1p/19q co-deletions, EGFR amplification and PTEN mutations) as compared to FISH.

Significance: With the development of this novel nCounter technique for clinical use, the study aims to determine whether nCounter technology can be a robust replacement for FISH in the genetic classification of brain tumours, leading to an efficient yet accurate measurement of CNVs as diagnostic, prognostic and predictive oncological markers. Ultimately, the diagnostic efficiency in laboratory analysis gained from this research work aims to improve the overall quality of health care approach in brain tumour oncology.

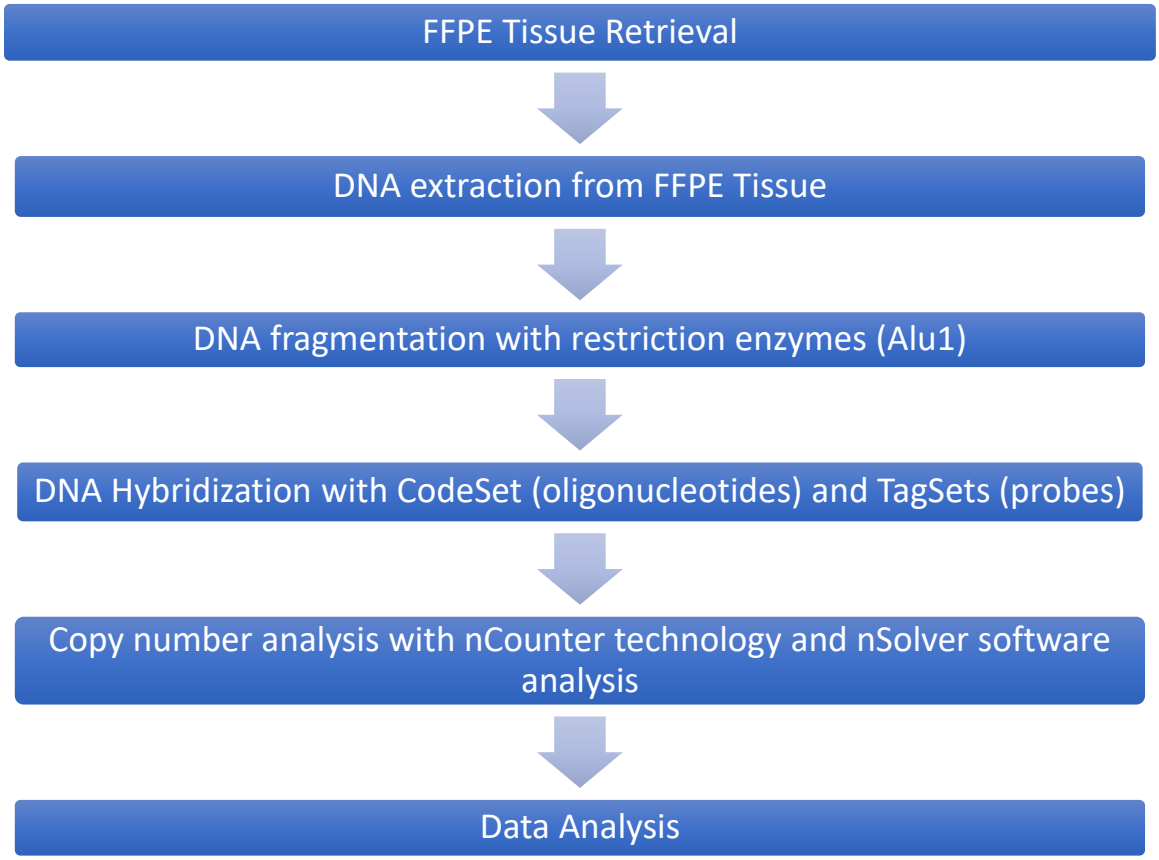
Ethics: Research ethics has been approved ("Molecular Analysis of Tumors of the Central Nervous System ", Pro00044306, PI Dr. F. van Landeghem). More specifically, the project aims to assess various method analysis parameters

Methods:

(1) Fluorescence in-situ Hybridization
   a. The fluorescence in-situ hybridization step was performed by diagnostic laboratories as per Alberta Health Services guidelines in brain tumour genetics.
   b. Subjects included in the study are as follows
      i. 1p and 19q co-deletion: 28 subjects
      ii. EGFR amplification: 20 subjects
   c. Brain tumour samples were collected during tissue biopsies performed by neurosurgeons at the University of Alberta Hospital, and were preserved for future analysis or resampling as formalin-fixed paraffin-embedded (FFPE) tissue.

(2) CNV Elements XT Assay

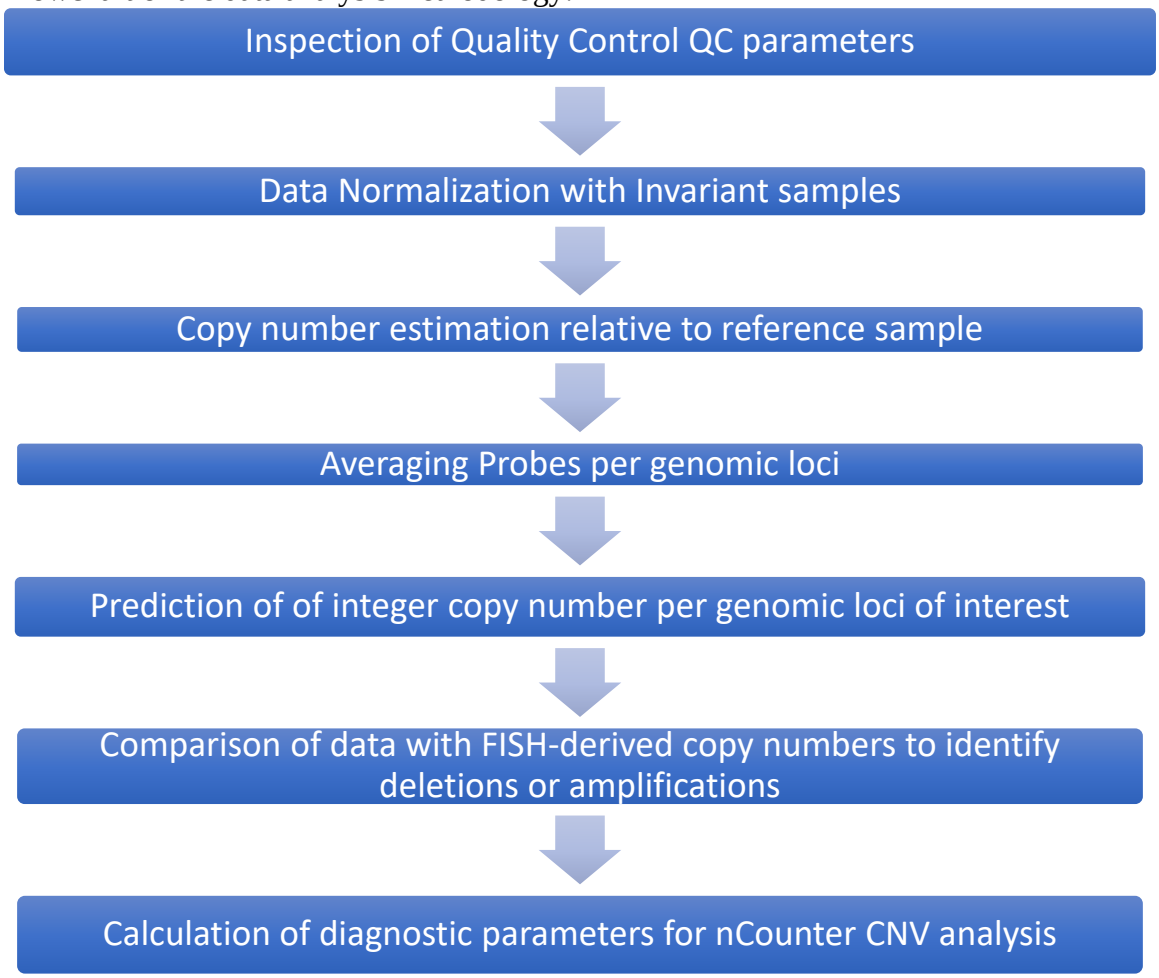
a. nCounter Assays with XT Formulations is the first step in the analysis of CNV changes in FFPE tissue. nCounter Elements XT technology can be used to detect DNA for purposes such as determining copy number variation (CNV) and performing counts of genetic loci in enriched DNA.
b. Steps in performing the experiment were based from the manufacturer's manual available online: See https://www.nanostring.com/application/files/1114/9212/1105/MAN-10023-11_nCounter_XT_Assay_User_Manual.pdf



(3) nCounter CNV Data analyses
a. nSolver Analysis Software version 2.6 was used to extract the RCC file sets post analysis. The latest version of Microsoft Excel was used to calculate and predict copy number estimates per genomic loci, and calculate diagnostic parameters for nCounter technologies. See the following guideline for the complete process in analyzing CNV data: https://www.nanostring.com/application/files/7114/8942/4663/MAN-C0014-02_nCounter_CNV_Data_Analysis_Guidelines.pdf
b. Several QC parameters measured in the study include:
    i. QC parameters
        1. Imaging QC (Field of View ratio and binding density QC): used to check for any errors during the imaging phase
        2. Control Probes QC: Positive Control Linearity QC: binding of probes to molecules added by nanoString

3. Positive Control of Limit of Detection QC: uses positive and negative control probes to confirm the limit of detection of each nCounter assay
4. Invariant Control QC: Each custom CNV assay contains a set of invariant control probes that target regions of the genome that have been measured as two copies in a majority of individuals.

c. Flowchart of the data analysis methodology:



(4) Comparison statistics

a. Several diagnostic parameters were used, including sensitivity, specificity, negative predictive value, positive predictive value, and likelihood ratios.
b. In order to calculate these parameters, comparison with the results from FISH analysis (as the gold standard) were done.
c. To indicate that a deletion (1p or 19q) were detected, a ratio of greater than 0.8 was used (similar to the guideline used by Alberta Health Services) with the signal measured from the same subject's 1q or 19p loci.
d. In *EGFR* detection, a positive deviation greater than + 2SD from the mean probe signal of all subjects was considered a positively detected amplification.

Results:

Table 1. Raw Data of nanoString detected results as compared with FISH results

Method	**1p deletion**	**19q deletion**	***EGFR* amplification**
FISH positive	9	8	4
FISH negative	19	20	16
True Positive	9	8	3
True Negative	8	10	15
False Positive	11	10	0
False Negative	0	0	1

Table 2. Summary of diagnostic parameters for nCounter CNV analysis as compared to FISH

Method	**1p deletion**	**19q deletion**	***EGFR* amplification**
Sensitivity (%)	100	100	75
Specificity (%)	42.1	50	100
Positive Predictive Value (%)	45	44.4	100
Negative Predictive Value (%)	100	100	93.8
Likelihood Ratio (+)	1.73	2.00	n/a
Likelihood Ratio (-)	0	0	n/a

Comparison of CNV nanoString assay versus FISH showed high concordance for EGFR amplification (3/4 positive and 15/15 negative) with specificity and positive predictive values of 100%. On the other hand, 1p or 19q deletion versus FISH showed high sensitivity (1p: 9/9 positive and 8/19 negative; 19q: 8/8 positive, 10/20 negative), with sensitivity and negative predictive values of 100%, but with lower specificity or positive predictive values ranging from 42-50%.

Discussion:

The diagnosis of brain tumours is critical in the determination of the prognosis and the approach to management of care for patients with brain tumours. In order for a robust diagnosis to be made, highly reproducible, specific and sensitive laboratory techniques are necessary to reduce the rates of brain tumour misclassification, as well as to increase the efficiency of reporting laboratory findings, and decrease tissue and DNA required to perform the analysis. More importantly, accurate and efficient molecular analysis on brain tumours will revolutionize the classification of gliomas in patients by implementation of an "integrated diagnosis" (Louis et al.). The purpose of this new approach—integrating histopathological, clinical and molecular findings into an integrated diagnosis— is a narrow, stringent definition of tumour entities and,

thus, an improved therapeutic stratification of brain tumour patients (Reuss et al.).

Copy number variations (CNVs) are structural changes at the DNA level, specifically with the normal repetitions of sequences, leading to either amplifications or deletions in the human DNA. An increasing number of studies have been linked CNVs to several diseases, including several cancers and schizophrenia. However, current technologies for CNV measurement are labor-intensive, offer low resolution in CNV analysis, and lack the ability to analyze multiple genes of interest concurrently per sample, creating a bottleneck in the screening for genetic oncological markers and diagnosis of brain tumours. Fluorescent *In Situ* Hybridization (FISH) is currently the gold standard and the most frequently used laboratory technique in measuring CNV because of its relative specificity and reproducibility.

Briefly, co-deletion of 1p/19q chromosomal arms typically indicate tumours of oligodendroglial origin and generally better prognosis and chemosensitivity (Boots-Sprenger et al.). On the other hand, *EGFR* amplification is supportive of a diagnosis of glioblastoma multiforme (GBM) or high grade astrocytoma, and predicts a poorer prognosis (Smith et al.). Having a robust and reliable method in detecting the molecular genetics behind brain tumours aids in the diagnosis and prognosis for each neuro-oncological patient.

Conclusion:

Our results support previous data from our group that copy number variation analysis by novel nCounter technology is a comparable alternative to the standard Fluorescence In Situ Hybridization (FISH) technique. Based on our results, detection of *EGFR* amplification is highly specific and is thus able to minimize false positives in molecularly diagnosing glioblastomas. Further, detection of 1p/19q deletion shows promising results, with 100% sensitivity, enabling FISH to minimize false negatives in predicting increased tumour chemosensitivity. Further assay optimization is necessary to improve false positive rates in the detection of chromosomal arm deletions and false negative rates in genomic amplification.

References:

Boots-Sprenger, Sandra H E et al. “Significance of Complete 1p/19q Co-Deletion, IDH1 Mutation and MGMT Promoter Methylation in Gliomas: Use with Caution..” *Modern pathology : an official journal of the United States and Canadian Academy of Pathology, Inc* 26.7 (2013): 922–929. Web.

Louis, David N et al. “The 2016 World Health Organization Classification of Tumors of the Central Nervous System: a Summary.” *Acta neuropathologica* 131.6 (2016): 803–820. Web.

Reuss, David E et al. “ATRX and IDH1-R132H Immunohistochemistry with Subsequent Copy Number Analysis and IDH Sequencing as a Basis for an ‘Integrated’ Diagnostic Approach for Adult Astrocytoma, Oligodendroglioma and Glioblastoma.” *Acta neuropathologica* 129.1 (2015): 133–146. Web.

Smith, J S et al. “PTEN Mutation, EGFR Amplification, and Outcome in Patients with Anaplastic Astrocytoma and Glioblastoma Multiforme.” *JNCI Journal of the National Cancer Institute* 93.16 (2001): 1246–1256. Web.

Impact of Award:

Being awarded a Brain Tumour Foundation of Canada Research studentship has been a huge honour. As a curious medical student trying to make contributions to medicine, being recognized and empowered to conduct research has had significant impact in my education so far. This project has immensely improved my ability to think scientifically, plan out experiments and be independent yet responsible in moving “my” project forward. There have been several roadblocks in this project, but that is something every researcher—much so students like me—encounters. But learning from our mistakes is integral to our growth as aspiring clinician-scientists. Not everything will turn out perfectly; not everything will always go our way. Realizing that early on in our training makes us adaptable and resilient, characteristics that are vital to someone aspiring to have a career in healthcare.

My sincerest gratitude goes to my supervisors Dr Frank van Landeghem and Dr Iyare Izevbaye from the Department of Laboratory Medicine and Pathology for their support, teaching and mentorship. Special mention goes to Kim Formenti within the Department, whose help was critical in the completion of this project. Lastly, my heartfelt gratitude goes to the Taite Boomer Memorial Brain Tumor Foundation and Brain Tumour Foundation of Canada, whose unwavering support to education and research is critical in the fight against brain tumours. Thank you all.