Reply to Wu and colleagues

Marc J. Williams[1-3,6], Benjamin Werner[4,6], Chris P. Barnes[2,5], Trevor A. Graham[1,*] & Andrea Sottoriva[4,*]

[1] Evolution and Cancer Laboratory, Barts Cancer Institute, Queen Marry University of London, London, UK.
[2] Department of Cell and Developmental Biology, University College London, London, UK.
[3] Centre for Mathematics and Physics in the Life Sciences and Experimental Biology (CoMPLEX), University College London, London, UK.
[4] Centre for Evolution and Cancer, The Institute of Cancer Research, London, UK.
[5] Department of Genetics, Evolution and Environment, University College London, London, UK.
[6] These authors contributed equally to this work.

[*] Correspondence should be addressed to T.A.G. (t.graham@qmul.ac.uk) or A.S. (andrea.sottoriva@icr.ac.uk)

Wu and colleagues' letter discusses the limitations of the use of single samples per tumor to investigate neutral evolution in human cancers. Neutral tumor evolution describes the situation where there is no differential clonal selection amongst the population of cells within a cancer: all mutations that accrue during growth are passengers and all drivers were already present in the first transformed cell.

In Williams et al., 2016[1] we showed, using publically available data comprising a single sample from each of ≈900 tumors of 14 different types, that the patterns of subclonal mutations within many cancer genomes (≈35%) were precisely predicted by a mathematical formula describing neutral tumor evolution. In contrast, Ling et al. 2015[2] performed high-density multi-region sequencing of a *single* hepatocellular carcinoma case (286 regions from a single tumor), and by examining the mutation burden across the tumor, concluded that the entire malignancy was evolving neutrally.

Wu and colleagues specifically question whether these two different approaches, namely analyzing intra-tumor heterogeneity within a sample versus dense multi-region sampling, measure the same features of tumor evolution.

Clearly, the key issue here is intra-tumor variation of the evolutionary process; specifically whether some regions of a tumor are evolving neutrality and others are not. We agree with Wu and colleagues assertion that 'local' neutrality (e.g. within a single sample) does not necessarily imply 'global' neutrality across the whole tumor.

However, there are two reasons to think that local and global neutrality are often correlated.

First, as we discussed in Williams et al., our classifications of neutrality were consistent with the detection of subclonal driver mutations in existing multi-region sequencing studies: sub-clonal driver mutations and convergent evolution (consistent

with ongoing selection) were often detected in 'non neutral-like' renal carcinoma[1,3] and glioblastoma[2,4], but less frequently in 'neutral-like' colorectal cancer[5].

Second, we note that if a single sample comprises a large portion or section across the tumor, neutrality can be assessed with our method based on the analysis of within-sample variant allele frequencies (mutations that are subclonal within the sample). Such a large sample can provide a global view of neutral evolutionary dynamics and, to degree with which a single large sample represents the tumor as whole, mitigates sampling bias. A similar approach has been successfully applied to deconvolute the clonal architecture of a single breast cancer case[6]. We note that the TCGA data we analyzed in our study is derived from large fresh-frozen resection specimens rather than small biopsies (http://cancergenome.nih.gov/cancersselected/biospeccriteria), thus reducing the sampling bias of our approach. However we fully acknowledge that no single sub-sampling strategy can fully capture the spatial architecture of a tumor and there is the need for extensive multi-region sequencing, which however remains at the moment impractical for large cohorts such as TCGA.

Importantly, as we noted in our study, the depth of sequencing remains a limitation as it determines the time elapsed from the common ancestor (of the sampled population) where we can investigate neutral evolution, as new mutations become progressively rarer as the population grows. We agree that for low depth of sequencing, only a short period after the common ancestor can be studied and so only 'global scale' neutrality (i.e. macroevolution) can be characterized, while the evolutionary dynamics of small populations (e.g. microevolution) remain inaccessible.

Given these two points, we think it is unlikely that our analysis risks grossly misrepresenting the tendency for neutral evolution in a tumor type.

While we fully agree that multi-region profiling reduces potential sampling bias (and indeed we use multi-region sequencing ourselves for this reason[6,7]), our method has the crucial advantage of allowing us to profile existing large cohorts (such as those of the ICGC and TCGA) and so to statistically address the issue of inter-patient variation[7] within a tumor type. Clearly the optimum would be to combine the two approaches and perform multi-region sequencing on large cohorts, though this presents obvious financial and technical challenges. We note too that studying truly 'local' evolution requires the sequencing of very small and localized cancer cell populations, as we previously demonstrated[5].

Wu and colleagues also note that non-exponential tumor growth leads to a different pattern of subclonal variant allele frequencies in a neutrally growing tumor: specifically boundary driven growth (described by $N(t) \sim t^{\gamma}$) leads to the relationship:

$$M(f) \sim \frac{1}{f^{\frac{\gamma}{\gamma-1}}}$$

which may provides a good fit to the data in some cases, and so neutrality may be more common than we reported in Williams et al. Irrespective, in some of the 65% of non-neutral cases identified by our method, clear subclonal mutational clusters can be observed, and our computational simulations confirmed that such patterns are expected if differentially selected subclones are present (Supplementary Figure 11 in Williams et al.). The observations of 'subclonal clusters' is in line with previous studies[6], and we note that amongst the TCGA samples we reanalysed in Williams et al., a previous analysis had detected subclonal peaks in the majority of cases[8]

(though we note this analysis may have confused the 1/f tail with a low-frequency clone). Thus, irrespective of the underlying growth model, there is clear evidence of on-going selection in many tumors.

However, we agree with Wu and colleagues that weak selection is challenging to detect because it causes only slight changes in the clonal composition of the tumor that may be undetectable by current genomic profiling standards. However this is true for single sampling and multi-region profiling alike. We acknowledge that it is very important at this stage to understand the precise signature of weak and strong of selection, especially because clonal selection is often hard to define and produces complex patterns (hence one of the reasons why we focused in the original manuscript on understanding *absence* of selection, which is analytically tractable). This important topic is the focus of our current and future work. Nevertheless, we note that the analysis in Williams et al. demonstrates that in a significant proportion of cases the null-model of neutral evolution cannot be rejected.

In summary, we were very happy to see that two independent groups have now demonstrated neutral evolution in cancer, a concept that has been largely neglected by current genomic studies. While the difference between local and global neutrality should be fully addressed in future work, the salient point that we would like people to take away from Williams *et al.* is that in many cancers neutral evolution is an entirely adequate description of the available data.

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Author Contributions

All authors contributed equally to this response letter.

Competing Financial Interests

The authors declare no competing financial interests.

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