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We read with great interest the commentary by Fraser[1], and wholeheartedly agree that understanding how selection acts on patterns of gene expression is key to identifying mechanisms of adaptive change. In Price et al[2], we identify significant challenges to testing how the transcriptome evolves, specifically, that shifts in tissue composition can bias inferences of selection over long evolutionary timeframes. However, Fraser[1] suggests the problems we outline have been 'largely solved by the research community' in two ways – through studies of cell lines and interspecific hybrids. We are in complete agreement that both approaches circumvent issues arising from varying cell type abundance that we highlight and so are useful tools to accurately measure expression change. However, neither represents a panacea for detecting natural selection on gene expression that Fraser[1] suggests.

Contrasts of cell lines can be used to accurately identify regulatory variation and in principle can be applied over greater evolutionary distances to quantify the mode of gene expression evolution among distantly-related species. However, creating cell lines is non-trivial and likely not feasible for many species. Importantly for multicellular model systems, the diversity of cell types that can be cultured is severely limited, and the costs in doing so prohibitive if all cell types in a tissue are to be included. This means that this approach is unlikely to extend across the tree of life in the near future.

Most importantly, organisms are far more than the sum of their parts. Changes in tissue composition are key to the evolution of many adaptive phenotypes (e.g.[3-6]) and likely the product of differences in expression across development[7]. Therefore, by their very nature, cell types analysed individually have limited potential for studying the developmental regulatory changes that produce variation in cell type abundance and complex adaptive traits. Consistent with these limitations, none of the vast cell line research cited by Fraser[1] tests for

selection on gene expression levels, with the exception of three studies using primate cell lines[8-10]. Examining cell types one at a time is therefore unlikely to yield a comprehensive picture of differences between species.

The second approach highlighted by Fraser[1] is the sign test of selection[11], which was extended by Fraser et al[12] to test for selection on gene expression. This method has provided important insight into how gene expression evolves[13], including the first known example of polygenic gene expression adaptation[12], and we have no wish to diminish this important contribution to the field. However, since this approach relies on prior knowledge of the directionality of genetic changes affecting a quantitative trait, to our knowledge, it has exclusively been applied to species that are able to produce viable hybrids, namely very closely related species[14-16] or subspecies[12,17,18]. Therefore, whilst informative for understanding expression evolution over very short evolutionary timeframes, it's potential to study many instances of adaptive change over the full breadth of evolutionary time is limited.

Together, neither approach suggested by Fraser[1] is widely applicable outside of model systems, limited cell types that are readily cultured, or relatively narrow evolutionary windows, leaving large gaps in both the scope of questions that can be addressed and the range of organisms that can be studied. Instead, we see a number of important points emerging from Fraser[1]. First, developmental context matters for the evolution of many adaptive phenotypes, particularly in multicellular organisms. For these traits we should focus not on eliminating differences in cellular composition but instead properly accounting for such differences when testing for selection on gene expression, potentially through the use of single-cell RNA-seq. Second, it is likely that selection pressures vary over short versus long evolutionary timeframes and limiting our analyses to closely related species will bias our understanding of how the transcriptome evolves. Comparative approaches that sample a range of evolutionary scales are clearly essential to understand the full spectrum of evolutionary responses to selection. Addressing the confounding issues of cellular composition, as discussed in Price et al[2], is therefore a major priority for the field.

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