

Whole Genome Microarrays and RNAi Tools and their Use to Elucidate Immune Signalling Pathways

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Introduction

Development and utilisation of functional genomic tools coupled with bioinformatic analyses in the malaria vector *Anopheles gambiae* have been central to our research. They facilitated generation of novel hypotheses that were subsequently tested experimentally by RNAi gene silencing and other molecular and cell biological methods. This approach led us to identify genes that are operational parasite antagonists or agonists, or participate in other mosquito innate immune reactions.

Tools for mosquito transcriptome studies were initiated on a first-generation microarray platform (4K chip) containing 4,000 ESTs prepared from two *A. gambiae* cell lines. These were used to explore the immune responses of cell lines and adult mosquitoes to diverse challenges (1, 2) as well as gene expression differences between malaria susceptible and refractory mosquito strains (3). More recently, our transcriptomic tool suite was extended to a new platform, 20K or MMC1 chip. Using MMC1 microarrays, we have studied mosquito midgut responses to invading malaria parasites (4) and defined the transcriptional programmes during mosquito development (5) and, in collaboration with other laboratories, explored additional aspects of vector biology such as insecticide resistance (6) and responses to viral infections (7). To support functional genomic approaches, we have developed an *Anopheles* EST informatics resource, AnoEST (8), which is presented separately.

Results and Discussion

We have generated a third microarray platform, MMC2, consisting of unique non-cross-hybridisable PCR amplicons designed by bioinformatics. The amplicons, which are 150-500 bp long, mostly represent single exons located preferably close to the 3' ends of coding sequences of 12,500 predicted genes in the *A. gambiae* genome. They carry bar-code sequences that alternate between neighbouring sequences in PCR plates and microarrays guarding against cross contamination. Also, they are in frame with the deduced proteins and include translation initiation and termination codons at the 5' and 3' ends, respectively, allowing synthesis of corresponding peptides which can be used for antibody production. Inclusion of bar-code sequences additionally permit the use of universal primer sequences during secondary amplification reactions that incorporate T7 promoter sequences which can be used for *in vitro* production of dsRNAs. A similar approach was used to develop DNA microarrays and functional dsRNAs in *Drosophila* (9, 10).

We aim to identify immune signalling pathways and networks using these tools. Our recent study of the Imd pathway in adult *Anopheles* using RNAi has revealed significant differences of the pathway functions between mosquitoes and fruit flies (11). In the mosquito, the pathway, which is responsible for immune activation of the antimicrobial peptide gene *CEC1*, is used for defence against both Gram-positive and Gram-negative bacteria and accounts partly for *Plasmodium* parasite losses in the mosquito midgut. Parasite killing at this stage is also achieved

through other immune pathways. These results urged development of approaches for the dissection of immune signalling pathways in a high throughput mode.

Towards this goal we are using complementary approaches. The first combines DNA microarrays and RNAi in characterised *Anopheles* cell lines. The use of cultured cells circumvents potential variations in silencing efficacy and regulatory pathways amongst adult tissues. Genes encoding putative pattern recognition receptors and other components of immune pathways are silenced, cells are then challenged with various immune elicitors and expression profiles are determined using the available DNA chips. To date, we have tested, optimised and preliminarily validated this experimental design using several genes. Parts of these studies will be presented.

The second approach utilise RNAi of the entire set of MMC2 dsRNAs in conjunction with reporter constructs to analyse classical signalling pathways, again in mosquito cell lines. Cultured cells are transfected independently with constructs in which reporter gene expression is driven by promoters of various immunity genes that are upregulated following immune challenge. DsRNA-treated cells are subsequently immune challenged and promoter activity is monitored. We recently began automated production of dsRNAs of the MMC2 amplicons and optimisation of the RNAi screens. Progress towards this direction will be discussed.

References

1. Dimopoulos, G., Christophides, G. K., Meister, S., Schultz, J., White, K. P., Barillas-Mury, C. and Kafatos, F. C. (2002) Genome expression analysis of *Anopheles gambiae*: responses to injury, bacterial challenge, and malaria infection. *Proc Natl Acad Sci U S A* 99, 8814-8819.
2. Christophides, G. K., Zdobnov, E., Barillas-Mury, C., Birney, E., Blandin, S., et al. (2002) Immunity-related genes and gene families in *Anopheles gambiae*. *Science* 298, 159-165.
3. Kumar, S., Christophides, G.K., Cantera, R., Charles, B., Han, Y.S., Meister, S., Dimopoulos, G., Kafatos, F.C. and Barillas-Mury, C. (2003) The role of reactive oxygen species on *Plasmodium melanotic* encapsulation in *Anopheles gambiae*. *Proc Natl Acad Sci U S A*, 100, 14139-14144.
4. Vlachou, D., Schlegelmilch, T., Christophides, G. K. and Kafatos, F. C. (in press) Functional genomic analysis of midgut epithelial responses in *Anopheles* during *Plasmodium* invasion. *Current Biol*
5. Koutsos, A. C., Blass, C., Zdobnov, E., Meister, S., Collins, Benes, V., F. C., Kafatos F. C., and Christophides, G. K. (2005). *Anopheles* microarrays and their use for comparative analysis of dipteran development. EMBO Workshop on Molecular and Population Biology of Mosquitoes and Other Disease Vectors, 24 - 31 July 2005, Kolymbari, Crete, Greece.
6. Vontas, J., Blass, C., Koutsos, A. C., David, J.-P., Kafatos, F. C., Louis, C., Hemingway, J., Christophides, G. K., and Ranson, H. (in press). Gene expression in insecticide resistant and susceptible *Anopheles gambiae* strains constitutively or after insecticide exposure. *Insect Mol Biol*
7. Sim, C., Hong, Y. S., Vanlandingham, D., Christophides, G. K., Kafatos, F. C., Higgs, S. and Collins, F. H. (in press) Gene expression profiling of the *Anopheles gambiae* response to *O'nyong nyong* virus infection. *Insect Mol Biol*
8. Kriventseva, E. V, Koutsos, A. C., Blass, C., Kafatos, F. C, Christophides, G. K., and Zdobnov, M. E. (in press). AnoEST: toward *Anopheles gambiae* functional genomics. *Genome Res*
9. Hild, M., Beckmann, B., Haas, S. A., Koch, B., Solovyev, V., Busold, C., Fellenberg, K., Boutros, M., Vingron, M., Sauer, F., Hoheisel, J. D. and Paro, R. (2003) An integrated gene annotation and transcriptional profiling approach towards the full gene content of the *Drosophila* genome. *Genome Biol* 5, R3.

10. Boutros, M., Kiger, A. A., Armknecht, S., Kerr, K., Hild, M., Koch, B., Haas, S. A., Consortium, H. F., Paro, R. and Perrimon, N. (2004) Genome-wide RNAi analysis of growth and viability in *Drosophila* cells. *Science* 303, 832-835.
11. Meister, S., Kanzok, S. M., Zheng, X. -L., Luna, C., Li, T. -R., Hoa, N. T., Kafatos, F. C., White, K. P., Christophides, G. K., and Zheng, L. (in preparation). The Relish/Imd immune pathway of the malaria vector *Anopheles gambiae*.